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A BIOCHEMICAL AND MORPHOLOGIC STUDY OF MYELINATION AND DEMYELINATION—III

EFFECT OF AN ORGANO-PHOSPHORUS COMPOUND (MIPAFOX) ON THE BIOSYNTHESIS OF LIPID BY NERVOUS TISSUE OF RATS AND HENS

GUIDO MAJNO and MANFRED L. KARNOVSKY
Department of Pathology, Department of Biological Chemistry,
and the Biophysical Laboratory, Harvard Medical School,
Boston, Massachusetts

(*Received* 25 *November* 1960)

THE HISTOPATHOLOGY of 'ginger paralysis', that is, of the central and peripheral lesions caused by organo-phosphorus compounds, has been described in considerable detail (SMITH and LILLIE, 1931; BARNES and DENZ, 1953; CAVANAGH, 1954). Biochemical investigations of this condition have focused largely on the concomitant inhibition of cholinesterases (ORD and THOMPSON, 1952; EARL and THOMPSON, 1952; EARL, THOMPSON and WEBSTER, 1953; DAVISON, 1953; ALDRIDGE, 1953 *a,b,c*; AUSTIN and DAVIES, 1954); less attention has been given to disturbances of lipid metabolism in the nervous tissue (WEBSTER, 1954; MAJNO and KARNOVSKY, 1955; AUSTIN, 1957; MAJNO and KARNOVSKY, 1958 *a*). This aspect seems worthy of further investigation, since the organophosphorus compounds have been referred to as 'myelin poisons' (SMITH and LILLIE, 1931). Their primary site of action, however, has not yet been conclusively established. Previous studies of this series have dealt with lipid biosynthesis *in vitro* by various preparations of central and peripheral, normal, and pathologic nervous tissue. The incorporation of labelled acetate and phosphate into the total lipids of these preparations was found to be a sensitive indicator of tissue damage even before the appearance of histological lesions in Wallerian degeneration, in the proximal stump of transected nerves (MAJNO and KARNOVSKY, 1958 *b,c*), in experimental diphtheric polyneuropathy (MAJNO, WAKSMAN and KARNOVSKY, 1960), and in central tracts after partial chordotomy (KARNOVSKY and MAJNO, 1959). In the present study the same techniques were applied to nervous tissue of rats and hens treated with Mipafox (*bis*mono*iso*propylaminofluorophosphine oxide). This substance, a powerful cholinesterase inhibitor, is used commercially as an insecticide; it is known to cause the typical paralytic syndrome in man and experimental animals (BARNES and DENZ, 1953).

MATERIALS AND METHODS

Animals. The rats were males from the Sprague-Dawley, Wistar, and Hisaw strains, maintained on Purina chow. The hens were pullets of the Rhode Island Red strain, all of one breed and approximately a year old. They weighed 2500–3500 g and were fed Purina laying mash. The animals were not starved before being killed, unless otherwise stated.

Tissue preparations. Rat nerves were the intact sciatic nerve (including the tibial branch) and the brachial plexus. Rat grey matter was represented by the first slice of cerebral cortex, approx. 350 μ in

thickness, cut with a hand microtome (MAJNO and BUNKER, 1957). A detailed description of these preparations was given in the first paper of this series (MAJNO and KARNOVSKY, 1958 *b*). White matter and sciatic nerves were also taken from hens. Usually the hen was killed by decapitation, the cord was exposed, cut just beyond the lumbar intumescence (24–25 cm), excised, deprived of the dura, and cut into 7 segments of the following lengths (in cranio-caudal direction): 10, 15, 70, 15, 70, 15 mm; the remaining portion and the first segment were discarded. The two longest segments (hereafter referred to as cervical and thoracic) were placed on chilled moist filter paper, and the anterior and lateral funiculi were stripped off to constitute four samples: 'cervical anterior', 'cervical lateral', 'thoracic anterior', and 'thoracic lateral'. Thus, each sample was composed of 2 homologous funiculi (left and right: 70–80 mg). The short segments were usually placed in neutral formalin for histological examination. The lowest (sixth) sample was just cranial to the lumbar intumescence. The sciatic nerves of the hen were exposed by excising the musculature; the trunk appeared subdivided into two parallel branches of approximately equal diameter, embedded in abundant adipose tissue. The epineurium of each branch was slit open with a pair of fine scissors, the nerve bundle was rolled out, and a segment 40–50 mm in length was excised. Histological examination showed this preparation to be free of endoneurial adipose tissue. The two pooled segments weighed 80–100 mg and were usually incubated as one sample. When six samples were prepared from one hen—four of white matter and two of sciatic nerve—the delay between death of the hen and the first manometric reading (see below) was 50–55 min, during which time excised tissues were chilled, then gassed with O_2 and equilibrated at 37°.

Incubation procedures. All tissues were incubated in the usual phosphate-buffered Krebs–Ringer medium, at 37° for rat and 41·5° for hen tissues with glucose as a source of energy (10 μmoles/ml). The radioactive substrate was placed in the side-arm and tipped into the main chamber 10 min before the first manometric reading. Subsequent readings were taken at 30 min intervals. After 4 hr the tissues were rinsed and homogenized in 2:1 chloroform–methanol. The lipids were extracted and washed four times by the method of FOLCH *et al.* (1951, 1957) and counted in a gas-flow counter. These techniques have been previously described in detail (MAJNO and KARNOVSKY, 1958*b*).

Radioactive substrates. The labelled lipid building-blocks were [1-^{14}C]acetate obtained from the New England Nuclear Company, [^{32}P]phosphate obtained from Oak Ridge, and [1:2-^{14}C]choline obtained from the California Research Corporation.

Mipafox [*bis*mono*iso*propylaminofluorophosphine oxide] was obtained through the courtesy of Mr. E. F. EDSON of Pest Control Ltd., Bourne, England; later it was purchased from L. Light and Co., Colinbrook, Nr. Slough, Bucks., England. It consists of transparent, very hygroscopic crystals. These were dissolved in distilled water to a concentration of 5 mg/ml and injected subcutaneously as described below. The solutions were made up immediately before use because the drug is rapidly hydrolysed to innocuous breakdown products.*

EXPERIMENTAL PROCEDURE AND RESULTS

Experiments on rats

Study of whole nerves from Mipafox-treated rats. The dosage of Mipafox was selected on the basis of data on the acute toxicity.* With the intraperitoneal route, the LD_{50} for male Wistar rats is 25–40 mg/kg. Females are reported to be more resistant: 23 daily intraperitoneal doses of 4 mg/kg cause no apparent adverse effects and the weight gain remains normal.* In our first experiment six male Wistar rats weighing 230–285 g were given four subcutaneous injections of 5 mg each (about 20 mg/kg) spaced 5–6 days apart. The animals showed occasional fasciculation of skeletal musculature a few minutes after the injection, but otherwise the drug was well tolerated. These rats were killed on the 23rd day (3 days after the last injection) along with 6 controls. The brachial and sciatic nerves of each animal were pooled as one specimen, and incubated with [1-^{14}C] acetate for 2 or 4 hr (Table 1). After 4 hr the nerves

* Data obtained from Medical Department, Pest Control Ltd.

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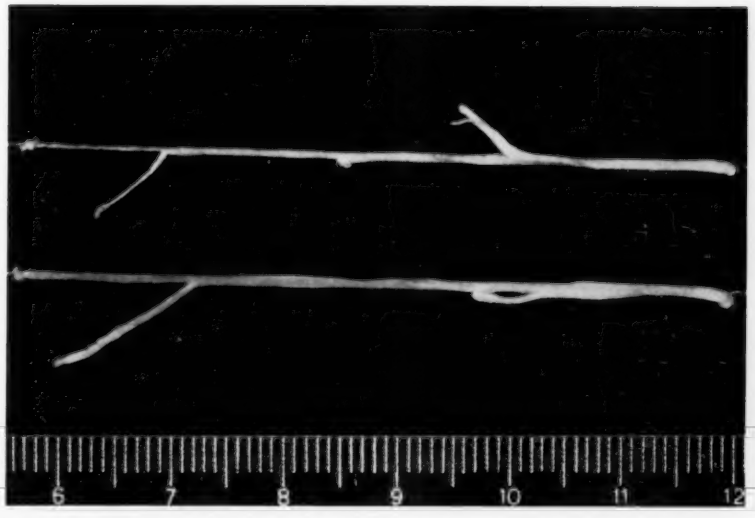
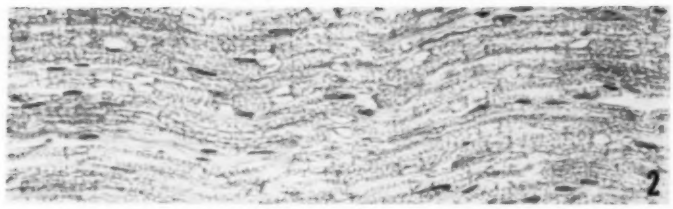
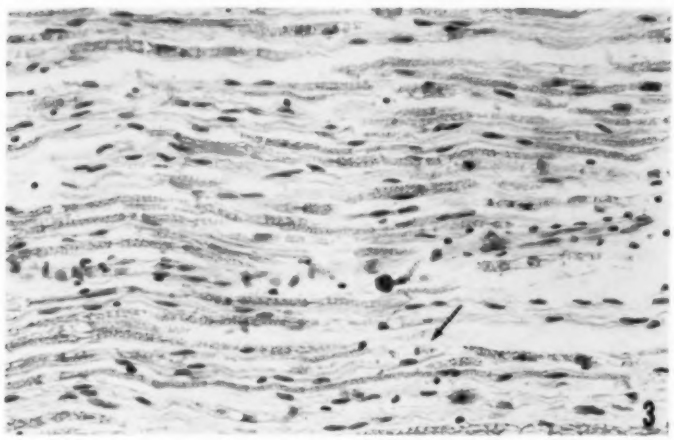
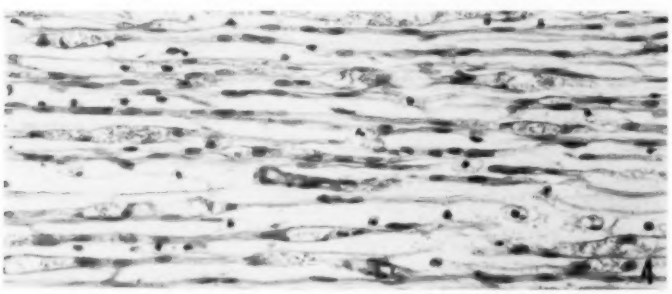


FIG. 1.—Gross aspect of the peripheral neuropathy induced by Mipafox in the rat. *Lower*: normal sciatic preparation, including the tibial branch (the side-branch is the peroneal). *Upper*: preparation from a rat of the same age and weight, injected with Mipafox twice weekly for 4 months (see text). The proximal portion is identical with that of the control; the distal is thickened, its greyish translucent aspect corresponding to the loss of myelinated fibres. Scale in mm.







FIGS. 2–4.—Rat sciatic nerves, fixed in formalin, embedded in paraffin, cut to 6 μ and stained with haematoxylin and eosin.

FIG. 2.—Proximal portion of the sciatic from a rat which received 237 mg of Mipafox in bi-weekly injections over 131 days. No lesions histologically demonstrable. Biochemically, preparations of this kind showed a decreased ability to incorporate acetate into lipids (compare with Fig. 5).

FIG. 3.—Distal portion of the same nerve as in Fig. 2 (tibial branch). Many fibres are replaced by 'Schwann cords,' and there is considerable widening of the interstitial spaces. Other fibres are still morphologically intact, as indicated by their 'neurokeratin' network. Few digestion chambers (arrow).

FIG. 4.—A 12-day stage Wallerian degeneration for comparison with the above figures.

of the injected animals had respired 18 per cent less than the controls, and the incorporation of acetate into the total lipids was 44 per cent of normal. Histologically, no lesions were found.

Statistical analysis of the data from this experiment (Table 1) left little doubt as to the validity of the results. However, three attempts to duplicate them remained unsuccessful, in that no significant differences between treated and untreated rats could be found. We are aware of only one factor which could have introduced a difference between the first and the subsequent experiments: a new batch of crystallized Mipafox had been used, and this had proved to be less potent in a test of acute toxicity. Furthermore, as will be seen below, there is a relatively advanced stage in the development of the neuropathy when no changes should be expected in respiration and lipogenesis from acetate if the entire length of nerve is studied.

TABLE 1.—EFFECT OF MIPAFOX TREATMENT ON RAT NERVES*

Incubation† period (hr)	Respiration (μl O_2)			Specific activity of lipid‡ (counts/min)		
	Normal	Treated	*P*	Normal	Treated	*P*
2	78·1§ ±3·1	68·3 ±2·6	<0·02	1000 ±71	613 ±81	<0·01
4	144·3 ±7·9	118·8 ±4·5	<0·02	1784 ±147	792 ±43	<0·001

* Four subcutaneous injections, each of 5 mg Mipafox were given 5–6 days apart, prior to carrying out the experiment *in vitro*.

† The sciatic nerves were incubated in Krebs-Ringer phosphate medium containing 10 μmoles glucose per ml and 1 mg sodium [1-^{14}C] acetate, equivalent to about 1 μc.

‡ Respiration as μl O_2 per 100 mg fresh tissue. Specific activity as counts/min per 100 mg lipid.

§ Twelve measurements were available for the respiration at 2 hr: six measurements were available for each of the other results.

The clue to this paradoxical situation was provided by three rats which continued to receive injections of this second batch of drug over a longer period (26 injections of 7 mg each). The animals remained in apparent good health, and their growth was identical with that of the controls. However, after about 3 months, it was noticed that two of them walked with paws somewhat abduced, reminiscent of the gait of the rat after sciatic section. The animals were killed on the 112th day (20 hr after the last injection) together with three controls. In two animals the tibial branch of the sciatic nerve had a very peculiar appearance: the distal 1/2 or 1/3 was swollen, pale, and somewhat translucent, closely resembling nerves in an advanced stage of Wallerian degeneration (Fig. 1). Incubation of the nerves (sciatics only) was carried out with [1-^{14}C] acetate; the respiratory CO_2 was also collected. This time each of the nerve preparations of the experimental group showed a higher lipogenesis from acetate than its paired control; oxidation of acetate to CO_2 was significantly increased, while oxygen uptake appeared unaffected (Table 2). Small samples of all nerves, proximal and distal to the incubated segment, were kept for histological study (Haematoxylin and eosin, scarlet red-haematoxylin, Marchi, Bodian method for axis cylinders). No lesions were found in the proximal segment; the distal part contained numerous degenerating fibres, as in a 2–3 week stage of Wallerian degeneration (Figs. 2, 3 and 4).

This experiment showed that Mipafox is capable of producing, in the sciatic nerve of the rat, gross and microscopic changes similar to those of Wallerian degeneration, and that these morphological changes appear first in the distal part of the nerve. Since the development of Wallerian degeneration is characterized by successive stages, during which respiration and lipogenesis from acetate first decrease, then increase (MAJNO and KARNOVSKY, 1958 *c*), it was surmised that similar polyphasic changes might occur along the sciatic nerve and branches after prolonged treatment with Mipafox. Hence, biochemical examination of this nerve should perhaps be performed

TABLE 2.—EFFECT OF MIPAFOX ON RAT NERVES (LONG-TERM TREATMENT*)

	Experiment	Control	Experimental
Oxygen uptake†	1	92·5	104·5
	2	113·5	109·5
	3	98·5	112·0
Oxidation of acetate to CO_2‡	1	1790	2692
	2	1820	2648
	3	1373	3059
Incorporation of acetate into total lipids§	1	1190	1240
	2	1130	1300
	3	775	1580

* 26 injections each of 7 mg Mipafox 4–5 days apart.
† μl O_2 per 100 mg fresh weight in 4 hr.
‡ Total counts as $BaCO_3$ in 4 hr, per 100 mg fresh tissue. When the means and standard errors were calculated, the experimental and control values differed significantly ($P < 0{\cdot}01$).
§ As specific activity, counts/min per 100 mg lipid. Although the results do not differ significantly on a statistical basis, each experimental animal gave a result higher than its paired control.

not on its entire length, but rather after subdivision into segments. The distal segment would be expected to represent the more advanced stages of the disease. This study was accomplished as described below.

Study of segmental differences along sciatic nerves of Mipafox-treated rats. The rats used for this study were submitted to long-term treatment with Mipafox so that the distal end of the nerve would develop relatively advanced lesions, while the proximal end would still be free from morphologically recognizable damage. Twenty-nine male Hisaw rats weighing 255–305 g were injected subcutaneously twice weekly. After three injections of 3 mg each, and three of 7 mg, the dose was maintained at 10 mg per injection. The growth curve remained indistinguishable from that of the controls. One rat died after 128 days. After 4–5 weeks the rats began to develop a sign which was found to be diagnostic of incipient neuropathy. The rat was placed on a flat surface, then lifted suddenly in mid-air by the tip of the tail. A normal rat will respond by an immediate extension of the hind limbs which tend to stretch along the axis of the body. Injected animals may fail to show this extension; in late stages they behave, in this respect, very much like rats with bilateral section of the sciatic nerve. It was also noticed that when the animals were lowered gently to the tabletop, normal rats reached the ground with the plantar surface of the hind paws, toes well extended; injected rats often failed to extend the toes and landed on the dorsal surface of their claws.

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Groups of rats were killed 40, 120, and 129 days after the first injection, when they had received a total dose of Mipafox amounting to 92, 237 and 362 mg, respectively. Upon dissection, the tibial nerves were found to be pale and swollen as already described, except for the 40-day group in which no gross lesions were recognizable. For biochemical examination the sciatic preparation—as previously defined—was arbitrarily subdivided into three parts: *distal* (the tibial nerve), *middle*, and *proximal* (the lower and upper half respectively of the sciatic trunk proper). This subdivision on the basis of anatomical criteria was maintained identically for all animals, irrespective of the extent of lesions recognized grossly. Segments from Mipafox-treated rats were

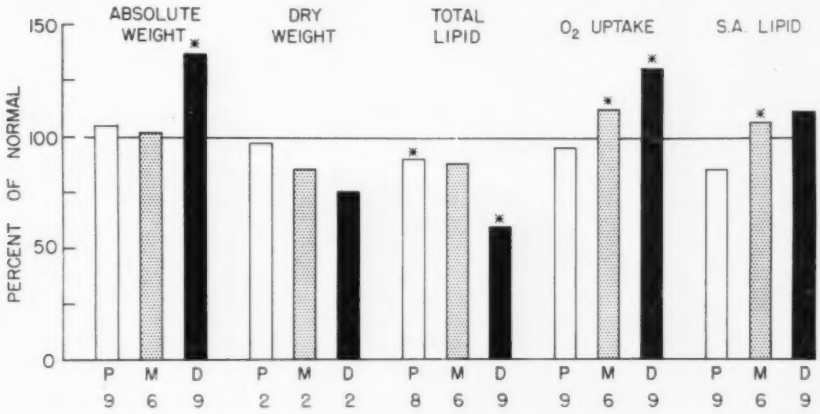

FIG. 5.—Effect of long-term Mipafox treatment on various segments of the rat sciatic preparation.
P: proximal segment. M: middle segment. D: distal segment. * represents a significance of $P < 0{\cdot}01$. The figures below the bars represent the number of animals for each determination.

incubated with [1-^{14}C] acetate and compared with homologous segments of controls. Nerves of comparable animals were kept for histological examination. In each experiment three flasks were prepared from each type of sample. Since proximal segments weighed considerably more than the middle and distal segments, the flasks were set up with either two proximal segments (one from each of two rats), four middle segments (from the same two rats) or four distal segments. The brachial plexus was incubated separately in triplicate (each flask contained one preparation from each of two rats).

Despite the different periods of duration of the treatment there was a broad overlap between the results obtained from each of the three groups of rats: hence the data were pooled (Fig. 5). At the proximal end of the preparation, in the injected animals, respiration and lipogenesis from acetate were somewhat depressed as compared with the normal; the distal end was characterized by an increase in absolute weight, it contained more water and less lipid than normal, and both respiration and lipogenesis from acetate were increased.

Effect of Mipafox on rat brain cortex. The purpose of this experiment was to study oxygen uptake and lipogenesis from acetate in grey matter of rats affected by Mipafox neuropathy. Twenty-four rats were injected according to the usual schedule, with a third batch of Mipafox purchased from Light and Co. After 3 months the 'kick' sign

was not yet demonstrable; hence the dose was increased to 15 mg per injection. At the end of the 5th month the rats were killed together with controls. Brain slices 0·35 mm in thickness were prepared with a microtome described earlier (MAJNO and BUNKER, 1957). The proximal and distal segments of the sciatic preparation were also taken. All samples were incubated with [1-^{14}C] acetate and compared with controls according to the usual plan (Table 3). Lipogenesis from acetate was equally depressed in the proximal and distal segments of the nerve, as might be expected for a very early lesion; an even greater depression occurred in the cerebral cortex (see Discussion).

TABLE 3.—EFFECT OF MIPAFOX ON INCORPORATION OF [1-^{14}C] ACETATE INTO LIPIDS OF RAT BRAIN CORTEX AND NERVE *in vitro*

Experiments		Normal	Experimental	*P*
Brain cortex*	4	100	54.5 ±8·8	<0·01
Sciatic, proximal	4	100	61·43 ±8·5	<0·01
Sciatic, distal	4	100	67·11 ±7·2	<0·01

* The results of each experiment were paired, the normal value being set at 100. The actual mean values (counts/min per 100 mg lipid) for the normal tissues were: Brain cortex, 22·9; Sciatic, proximal 46·3; Sciatic, distal 39·3. The values for proximal and distal segments of the sciatic nerve were not significantly different from each other.

Experiments on hens

The LD_{50} value of Mipafox for the hen (referred to acute, single-dose toxicity, gastric route†) is 30 mg/kg. We found that a paraplegic syndrome could be produced quite consistently by administering two subcutaneous doses of 10 mg/kg each, 2–8 days apart. Immediate effects referable to acute cholinesterase inhibition were observed in only one of 32 hens (lung oedema, transient). The first neurological symptoms usually appeared 12–14 days after the first injection (unsteadiness, tendency to sit for longer periods than the controls). By the 18th–22nd day most hens were incapable of standing; they sat with both hind limbs stretched to one side, and the weight declined rapidly. The wing muscles appeared to be relatively spared; occasionally the respiratory muscles were involved (dyspnoea). Each injected hen was paired with a control of comparable initial weight.

Effect of Mipafox injection: experiments with uncontrolled food intake. These hens were allowed food and water *ad lib.* and were killed 23–27 days after the first dose; by this time they had lost 33–40 per cent of their initial weight. Spinal white matter and sciatic nerves were incubated with [1-^{14}C] acetate (Table 4). Both cervical samples showed a 10–20 per cent depression in respiration. An interesting incidental observation was that all samples of anterior funiculus respired 20–30 per cent less than those of the lateral funiculus, both in the normal and in the treated hens. Lipogenesis from acetate in the sciatic nerves was depressed to less than half, and a clear cut fall occurred also in both cervical funiculi. In the thoracic segment there was a depression in the anterior funiculus and no significant change in the lateral. The morphological changes in both cord and nerves will be discussed further.

† Data obtained from Medical Department, Pest Control Ltd.

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Since the most striking results were obtained with the sciatics, an effort was made to establish the time of appearance of the biochemical lesion in this tissue. Sets of three hens were killed 3,6,9, and 14 days after the injection of Mipafox, and the sciatics were excised and incubated with [1-^{14}C] acetate together with controls. Oxygen uptake and specific activity of the lipids showed a greater scatter than normal, but no single trend emerged, hence these data are not presented in detail.

Up to this point, then, it was established that consistent biochemical lesions in the sciatic nerves were to be found only during the fourth week after the injection of the

TABLE 4.—EFFECT OF MIPAFOX ADMINISTRATION ON THE NERVOUS TISSUE OF HENS*

	Sciatics	Cord			
		Cervical		Thoracic	
		Anterior	Lateral	Anterior	Lateral
Respiration†	95·5 ±6·2	82·7 ±3·0	82·8 ±3·5	104·3 ±9·0	106·7 ±13·5
P	>0·5	<0·05	<0·05	>0·5	> 0·7
Lipids‡	43·5 ±9·3	60·5 ±10·6	53·8 ±6·3	72·3 ±9·0	129·0 ±18·5
P	<0·01	<0·05	<0·01	0·05	> 0·5

* Results expressed as percentage of normal values in each experiment. Two separate doses (10 mg/kg) of Mipafox were injected subcutaneously one week apart. Hens killed after 23–27 days. At this time the experimental animals had lost 33–40 per cent of their initial weight, while the controls were stationary.

† Expressed originally as μl O_2 per 100 mg fresh tissue per hr. Three experiments performed.

‡ Expressed originally as specific activity, counts/min per 100 mg lipid. [1-^{14}C] Acetate was the precursor. Four experiments were performed.

drug, at a time when the paralytic symptoms were already full-blown, and the general condition of the hens was rapidly deteriorating. This suggested that the results might reflect the poor nutritional state of the experimental animals rather than a direct effect of the drug itself. Hence, a series of experiments was set up in which the injected and control animals had, as closely as possible, the same nutritional condition.

Effect of Mipafox injection: experiments with controlled food intake. Six hens were paired by weight; of each pair one hen received two injections of Mipafox (1st and 8th day). All hens were allowed food and water *ad lib.* Weights were recorded daily. Changes during the first week were slight and somewhat erratic. During the second week all injected hens were losing 10–30 g/day, and the food allowance of the controls was reduced in an attempt to match their weights. At the 15th day the matching was satisfactory (Table 5) with the exception of the third pair, in which the injected hen was losing weight rapidly. Paradoxically, this was the only hen which appeared otherwise symptom-free; the others were beginning to show unsteadiness. From this day on all hens were force-fed twice daily by stomach tube (a rubber catheter connected to a syringe) with a commercial brand of evaporated milk (Klim) prepared at twice the concentration indicated by the manufacturers (136 g/500 ml: 50 ml at 9 a.m., 75 ml at 4.30 p.m.).§ Water was allowed *ad lib.* During the 7–10 days of force-feeding a weight loss was noticeable in the controls (3–5 per cent) but the injected animals lost weight

§ We are indebted to Dr. S. GERSHOFF of the Department of Nutrition, Harvard School of Public Health, for the suggestion of this method.

TABLE 5.—WEIGHT CHANGES OF PAIR-FED HENS (NORMAL AND MIPAFOX-INJECTED)

Hen	Experimental period (days)	Initial weight (g)	Loss in weight by 15th day (%)	Weight loss during forced feeding* (%)	Total weight loss (%)
Control 1	21	3380	1·8	5·2	7·0
Injected 1	21	3440	2·5	8·7	11·2
Control 2	23	2860	8·2	3·6	11·8
Injected 2	23	2950	8·8	7·7	16·5
Control 3	24	2570	8·5	2·0	10·5
Injected 3	24	2470	15	3·5	18·5

* Force-feeding started on the 15th day.

TABLE 6.—EFFECT OF MIPAFOX ADMINISTRATION ON THE NERVOUS TISSUES OF HENS, UNDER CONDITIONS OF CONTROLLED FOOD INTAKE*

	Sciatics	Cord†	
		Anterior	Lateral
Respiration‡	(1) 96·0	77·0	100·5
	(2) 93·5	76·0	96·0
	(3) 95·5	90·0	126·0
Lipids§	(1) 207	85	87
	(2) 220	95	130
	(3) 56	74	92

* Results expressed as percentage of normal values in each experiment.
† Only the cervical segment was studied. See text.
‡ Expressed originally as μl O_2/100 mg fresh tissue per hr.
§ Expressed originally as specific activity, counts/min per 100 mg lipid.

at about twice this rate (Table 5). The pairs were killed 21–24 days after the first injection; two hens had been paralysed for 3–5 days, the third could still stand, though unsteadily, on the day it was killed. On this day an unusual change was observed in the left sciatic nerve, and that only, of one injected hen: the anterior branch was so yellow that it blended with the surrounding adipose tissue. When stripped of the epineurium it appeared greenish-yellow and punctuated with numerous petechiae; the other branch was unchanged. Histologically these nerves showed advanced lesions of a degree never found in previous experiments. Incubation of the tissues was carried out according to the usual scheme, except that only the cervical segment of the cord was sampled (Table 6). In the sciatic nerves, oxygen uptake was slightly depressed, as had been found in previous experiments; incorporation of acetate into the total lipids, on the other hand, was doubled in two cases, and depressed

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only in one animal. In the cervical anterior sample of spinal cord both functions were somewhat depressed, though to a lesser extent than was previously found; the cervical lateral sample behaved erratically (see Discussion).

It is clear, then, that the nutritional state of the animals has a considerable influence on the course of the biochemical changes found in Mipafox poisoning of the hen. In a further series of experiments the effects of starvation alone were studied.

Effects of starvation alone. In a preliminary experiment it was found that hens could be entirely deprived of food for a period of 9 days without apparent untoward effects but for loss of weight. Accordingly, 6 hens were submitted to complete starvation (with water *ad lib.*) for 7 or 8 days; each hen was weighed daily, together with a hen

TABLE 7.—EFFECT OF STARVATION ON RESPIRATION AND LIPOGENESIS OF HEN NERVOUS TISSUE *in vitro**

Precursor	Oxygen uptake†				Lipid specific activity†			
	Nerves	*P*	Cords	*P*	Nerves	*P*	Cords	*P*
[1-^{14}C] Acetate	87·5‡ ±2·9	<0·02	98·0‡ ±10·4	>0·8	39·6 ±13·6	<0·05	103·0 ±29·0	>0·5
[^{32}P] Phosphate					68·4 ±16·8	<0·05	98·5 ±6·2	>0·8

* Animals were starved 1 week, but water was allowed *ad lib.* Compared with fed controls, whose weight remained essentially stable, the starved hens lost 13–27 per cent (average 17·5 per cent) of their weight.

† The data are expressed as a percentage of the normal value in each experiment.

‡ The data from both labelling experiments are combined. Five results were obtained in the case of respiratory measurements: three experiments were carried out for each labelled substrate.

of comparable initial weight which was kept on a standard diet and served as a control. The weight of the controls remained approximately stationary (there was an average loss of 2·5 per cent). The starved birds lost 13–27 per cent (average 17·5 per cent) while their general condition remained surprisingly good: after 1 week they appeared as alert as the controls. The stomach contents at this time consisted of a few pebbles and bile-stained sawdust.

The hens were killed on the 7th day of starvation together with their individual controls. Two segments of white matter (anterior funiculus) were taken from each spinal cord: one was incubated with [1-^{14}C] acetate, the other with [^{32}P] phosphate. The remaining hens were killed on the 8th day, and their sciatic nerves were excised; the left was incubated with [1-^{14}C] acetate, the right with [^{32}P] phosphate (Table 7).

For the white matter there was no measurable difference between experimental animals and controls; in the sciatic nerves, on the other hand, there was a 13 per cent decrease of respiration and even greater depressions in the incorporation of acetate and phosphate into the lipids.

DISCUSSION

The diseases induced in hens and rats by Mipafox, while fundamentally similar, follow very different courses. In the hen, a relatively small dose leads to severe paraplegia within 2 weeks; the histopathologic changes have been carefully described by BARNES and DENZ (1953) and by CAVANAGH (1954). Rats tolerate higher doses

every week for several months without deviating from their normal growth curve; the only symptom which may develop is a slight impairment in the use of the hind limbs (BARNES and DENZ, 1953). Because of this apparent resistance (MENDEL and MYERS, 1952) rats have not been used for the study of demyelination caused by organophosphorus compounds. Actually, our results with Mipafox-injected rats show that the peripheral neuropathy, though slow in developing and almost asymptomatic, may attain gross and microscopic features even more pronounced than those observed in the hen. Furthermore, the slow, steady course of the neuropathy in the rat offers certain advantages for a biochemical study of the nerve. In the first place, the general condition of the rat remains excellent; hence there is no interference by a superimposed nutritional disturbance as occurs with the hen. Secondly, the morphological lesion progresses from the peripheral to the central end of the nerve at such a slow pace that the distal half of the tibial branch may be severely damaged while the proximal half of the sciatic trunk is still morphologically intact. This makes it possible to subdivide the nerve into segments, each containing a population of fibres which are at relatively similar stages of disease (Figs. 2 and 3).

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Effect of Mipafox on the sciatic nerves of the rat

A striking feature of the neuropathy in the rat is the gross aspect of the tibial nerve, which may become so swollen and pale that the existence of the neuropathy may first be recognized by the naked eye (Fig. 1). The swelling is related to an increase in the content of water (Fig. 5) and it is very similar to that encountered at the height of Wallerian degeneration in the rat (MAJNO and KARNOVSKY, 1958*c*). The histologic appearance suggests, in both conditions, that most of the fluid is accumulated in the extracellular spaces (Figs. 2, 3, and 4). We do not know the laws which regulate the processes of vascular filtration and reabsorption in nerves; it has been established, however, that there is a 'blood-nerve barrier' (WAKSMAN, 1960) which under normal conditions in the rat does not allow trypan blue, injected intravenously, to appear in appreciable amounts inside nerve trunks. In Wallerian degeneration this 'barrier' apparently breaks down, so that the dye accumulates in the endoneural fluid (Fig. 6). The same appears to be true for the Mipafox neuropathy; here the blueing is limited to the swollen segment (Fig. 6). It would seem that, as the nerve fibres disappear, the surrounding vessels tend to become similar, in their functional behaviour, to those of ordinary connective tissue.

BARNES and DENZ (1953) kept rats for 3 months on a diet containing 300 p.p.m. Mipafox. The resulting lesions were very mild, but the dosage was probably lower than that employed by us. In our own experiments we observed considerable variations in activity between batches of Mipafox. No attempts were made to purify the substance further, but it is possible that these variations may be related to the instability of the drug, which is hygroscopic and easily hydrolysed.* Though all batches were effective to some degree, there were variations in acute toxicity as well as in the capacity to induce peripheral neuropathy.

Our biochemical data may be summarized as follows: the earliest biochemical change brought about by Mipafox in the rat's sciatic nerve (prior to the development of histological lesions) is similar to that found in early Wallerian degeneration, namely, a depression of respiration and of lipogenesis from acetate. This can be seen after

* Data obtained from the Medical Department of Pest Control Ltd.

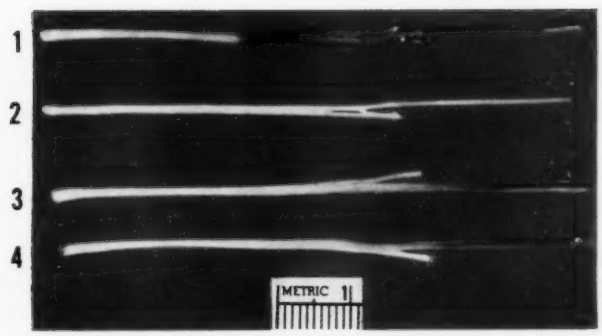

FIG. 6.—Demonstration of the breakdown of the 'blood-nerve barrier' in Mipafox neuropathy and in Wallerian degeneration. Rat sciatic preparations, stripped of the epineurium. All the nerves were excised from rats which received a daily intravenous injection of trypan blue 1% (0·5 ml per 100 g body weight) on the 3 days preceding sacrifice.

(1) Sciatic which was transected 31 days previously. Note swelling and accumulation of dye distal to the site of operation.

(2) Intact, contralateral nerve of the same rat. No appreciable amount of dye has leaked into the nerve.

(3) and (4) Nerves from a Mipafox-treated rat (2 weekly injections over 4 months). Dye has leaked into the distal portion of the nerve, where swelling is also apparent. The histological appearance of the proximal and distal portions of such a nerve are shown in Figs. 2 and 3. In nerves 1, 3 and 4 the dye is largely extracellular, as indicated by the fact that it tends to leach out quite easily if the nerves are laid on moist filter paper.

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long-term injection at the proximal end of the nerve when the distal end is already in an advanced stage of the disease (Fig. 5). The same disturbance was also found along the whole nerve in rats which had been treated for 5 months with a batch of Mipafox exhibiting low toxicity. In a later stage, the nerve is characterized by a somewhat increased respiration and specific activity of nerve lipid ([1-^{14}C]acetate experiments), changes which are also characteristic of advanced Wallerian degeneration in the rat (MAJNO and KARNOVSKY, 1958*c*).

Effect of Mipafox on peripheral nerves of the hen

The metabolic data obtained from the hen were less satisfactory. In fact, as will be discussed below, a number of difficulties were encountered in working with these animals.

In the latent stages of the disease and up to the time of paralysis (14 days) all we could detect in the sciatic nerves, with regard to respiration and lipogenesis from acetate, was a broad scattering of results with no obvious pattern. In later stages, after the onset of paralysis, both respiration and lipogenesis were depressed, but it soon appeared that these animals did not feed properly and that the effects of starvation were complicating those of the drug. When the hens were force-fed, lipogenesis from acetate proceeded at twice the normal rate in two animals and at half this rate in the third; we interpret this scatter as reflecting the varying admixture of early and late stages of degeneration apparent in the tissue sections. Respiration, however, remained significantly depressed (Table 6). In general, then, the biochemical lesions found in hen nerves—though less consistent—were similar to those found in the rat.

A few pertinent data, also rather fragmentary, are available from the literature. HELLER and HESSE (1959) found a decreased endogenous respiration in the sciatic nerves of paralysed hens, 12–19 days after the injection of triorthocresyl phosphate (TOCP). These authors failed, however, to control the possible effect of malnutrition. AUSTIN (1957) found a 20 per cent depression of lipogenesis from acetate in hen sciatics, 24 hr after the injection of Dyflos (DFP; di*iso*propylphosphorofluoridate), while at 5 and 17 days this depression was no longer significant. WEBSTER (1954) studied the uptake of [^{32}P] phosphate by the sciatic nerves and cords of hens injected with TOCP. The labelled phosphate was administered *in vivo* 4 and 14 days after the injection of TOCP; 3 days later there was no difference in any of the fractions examined.

In reviewing these different studies of 'ginger paralysis' in hens, it is rather striking to notice that biochemical functions are often reported as apparently unaffected in tissues which showed histological evidence of damage. We should like to emphasize that this lack of change may only be apparent. It was already mentioned that certain functions are depressed in the early stages of Wallerian degeneration, and increased later; if fibres in both stages are present in a given nerve, and this is assayed as a whole, the overall result may be an 'average' in which changes in opposite direction are cancelled out. This is not so likely to occur in the rat sciatic, in which the neuropathy proceeds very slowly from the distal to the proximal end of the nerve; in the hen, far more susceptible to the drugs of this group, the fibres degenerate more rapidly and not simultaneously; hence the proximo-distal pattern might be less obvious. Its existence was, in fact, recently denied (SILVER, 1959).

The hen is ordinarily the animal of choice for the study of 'ginger paralysis', because of its characteristic susceptibility. Actually, a number of reasons operate to

make it very difficult to obtain satisfactory, comparable samples of sciatic nerves from these animals. Individual differences, in our experience, are considerably greater than with rats. Also individual birds vary considerably in their sensitivity to the drugs (see also BARNES and DENZ, 1953). Furthermore, the rapidly progressing neuropathy does not necessarily affect to an identical degree the two symmetrical nerves, or even the two branches which compose the sciatic trunk; these branches, in fact, are known to differ considerably in their oxygen uptake under normal conditions (HELLER and HESSE, 1959).

Effect of Mipafox on central tracts (*white matter*)

This study was limited to hens, because the number of degenerating fibres in the *funiculus gracilis* of chronically treated rats was too small to allow biochemical studies.

In the white matter of hens which were injected with Mipafox and not force-fed, there was a major depression of lipogenesis in three of the four selected samples (Table 4). When the hens were force-fed after injection of Mipafox, a depression of both functions remained significant in one sample only, that which contained the greatest number of degenerating fibres (Table 6). The data therefore suggest that Marchi-positive fibres have a decreased ability to synthesize lipid from acetate.

The negative finding on the other samples—which presumably also contained some degenerating fibres—is probably not to be taken at face value. The excision of degenerating tracts from the hen's spinal cord is necessarily 'blind' because there is no gross alteration to use as a guide. Recent studies on the posterior funiculus of the rat after partial chordotomy have provided more reliable samples of degenerating central tracts (KARNOVSKY and MAJNO, 1959). Lipogenesis from acetate dropped rapidly and remained depressed even in later stages, up to 100 days after the transection. This probably reflects the lack of regenerative power characteristic of central tracts, and the lack of a cellular reaction comparable to the hypertrophy and hyperplasia of the Schwan cells.

Effect of starvation alone. It is obvious from the preceding paragraphs that starvation plays a major role in enhancing the metabolic changes of 'ginger paralysis'. As for starvation alone, peripheral nerves were found to be—as expected—more sensitive than white matter; respiration and the incorporation of acetate and of phosphate were all found to be depressed, though quantitative differences may be found between the effects on hens (Table 7) and guinea pigs (MAJNO, WAKSMAN and KARNOVSKY, 1960). Despite the surprising resistance to starvation exhibited by the white matter of *normal* hens (Table 7), starvation appeared to enhance the central lesions of Mipafox-treated hens (Table 4).

It is pertinent to review the available data on the relative changes of acetate and phosphate incorporation into nerve lipid under pathologic conditions. The overall pattern which seems to emerge is that the incorporation of acetate is easily depressed and thus represents a more sensitive indicator of regressive changes in the myelin* (or Schwann cell); the incorporation of phosphate can also be depressed, but it rises very easily with progressive changes in the Schwann cells (hypertrophy, hyperplasia) hence it is a more sensitive indicator of reactive and presumably reparative changes in these cells. Thus, in all pathologic conditions to be enumerated, there is always an

* Strictly speaking 'nerve lipid' would be a more appropriate expression.

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initial depression of lipogenesis from acetate, whereas the incorporation of phosphate drops or rises, depending on the condition of the Schwann cells. For instance, in Wallerian degeneration, in which the Schwann cells are presumably normal and promptly stimulated to hypertrophy and hyperplasia, there is also a prompt rise in phosphate incorporation. In diphtheric polyneuropathy there is a primary damage to the Schwann cells, expressed morphologically by the segmental type of demyelination; there is also an initial drop in the incorporation of phosphate. The same is true for starvation; when lesions are found, they are of the segmental type (see DENNY-BROWN, 1947; SWANK, 1940), which indicates an injury to the Schwann cells; again, there is a depression of the incorporation of phosphate, at least in the rat and hen. In guinea pigs submitted to partial food restriction for intervals up to 16 days there was no significant change (MAJNO, WAKSMAN and KARNOVSKY, 1960). When nerves are incubated and therefore allowed to degenerate *in vitro*, the resulting morphological changes are not dissimilar to those of Wallerian degeneration, but there is no Schwann cell reaction, and the incorporation of phosphate drops (MAJNO and KARNOVSKY, 1958*c*).

It is obvious from the preceding discussion that a drop in lipogenesis from acetate is a precocious, but very non-specific, sign of injury to the tissue. So far it has been found, at one stage or another, in every neuropathy in which it has been sought. Hence there is little justification for considering it the primary lesion caused by any specific poison, such as Dyflos (AUSTIN, 1957).

Pathogenesis of the lesions induced by Mipafox. Ever since the early descriptions of neuropathies induced by organo-phosphorus compounds, the pathogenesis of the characteristic lesions has been a subject for controversy. Early workers favoured the opinion that the drugs act as a 'specific myelin poison' (SMITH and LILLIE, 1931), the primary target being, therefore, the fibre itself rather than the corresponding cell body. The major arguments in favour of this interpretation are briefly the following (see also CAVANAGH, 1954): (1). The fibres show very prominent lesions, which are a constant feature of this group of diseases. (2). The pathologic changes in the perikaryon vary quite considerably in severity from one animal to another, independently of the severity of the peripheral lesion and of the paralytic symptoms (SMITH and LILLIE, 1931). (3). The lesion of the cell body is not necessarily primary; it could be explained as a secondary reaction to damage in the fibre, *i.e.*, as an 'axon reaction'. (4). Pseudocholinesterase is mainly associated with the fibres and the organo-phosphorus compounds are known to be inhibitors of this group of enzymes. Hence it was once suggested by EARL and THOMPSON (1952) that the demyelination may result from an inhibition of the pseudocholinesterase in the fibres. This argument, however, was withdrawn because subsequent work proved that the demyelination does not correlate with the inhibition of the pseudocholinesterase (THOMPSON, 1953), or even with the species distribution of this enzyme (MENDEL and MYERS, 1952).

The opposite view, namely, that the primary damage caused by organo-phosphorus compounds is in the perikaryon, relies upon the following arguments: (1). A segmental demyelination has never been found in these neuropathies. This condition, first described by GOMBAULT (see KRUECKE, 1955), represents a true demyelination in that the myelin breaks down along isolated segments of the fibre, presumably as an effect of damage to one or few Schwann cells, and without apparent involvement of the axon. If the organo-phosphorus compounds exerted a local noxious effect upon the myelin

sheath, some degree of segmental demyelination should be expected. (2). In the neuropathy caused by organo-phosphorus compounds, axon and myelin sheath appear to break down simultaneously (CAVANAGH, 1954). Again, if the lesion were primarily one of the myelin, one would expect the axon to be temporarily spared. (3). The simultaneous disappearance of axon and myelin suggests an analogy with Wallerian degeneration, in which axon and myelin break down because they are severed from the perikaryon. In the neuropathies caused by organo-phosphorus compounds a similar mechanism may be at play, in that the fibres may degenerate because the perikaryon becomes inadequate to sustain them.

In concluding, it is clear that there are no definitive arguments on either side, though the weight of the evidence seems to be in favour of a primary central lesion. Our own experiments are not decisive, but add circumstantial evidence in favour of a primary central lesion: *i.e.*, the biochemical similarities between the neuropathy induced by Mipafox and Wallerian degeneration and the existence of a biochemical defect in the brain cortex. Furthermore, some experiments were performed to test the effect of Mipafox on lipid biosynthesis by nervous tissue *in vitro*. The results will be published elsewhere; they show that Mipafox does inhibit the incorporation of acetate, choline, and phosphate into the lipids of nervous tissue, but at concentrations similar to those of non-demyelinating drugs; hence no evidence was obtained that Mipafox is a 'specific myelin poison'.

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SUMMARY

As part of a study of demyelination, rats and hens were injected with Mipafox (*bis*mono*iso*propylaminofluorophosphine oxide), a cholinesterase inhibitor known to produce a peripheral neuropathy in man. The drug was administered subcutaneously for periods ranging from 3 to 150 days. Subsequently the animals were killed, various preparations of nervous tissue were obtained and incubated in Warburg flasks in the presence of a radioactively-labelled lipid precursor ([1-^{14}C] acetate). After incubation the total lipids of the tissue were extracted and their radioactivity was determined in a gas-flow counter. Comparable samples were kept for histological examination.

In rats, the drug could induce, within 3–4 months, a typical peripheral neuropathy consisting of subtotal degeneration of the branches of the sciatic nerve, while the proximal portion of the sciatic trunks remained histologically intact. There was no loss in body weight. Respiration and lipid biosynthesis from acetate were depressed in the proximal portion of the sciatic nerve (where no lesions were demonstrable) and increased in the degenerating branches. These changes are similar to those previously found in early and late stages of Wallerian degeneration, respectively. The potency of the drug as a 'demyelinating' agent in rats varied somewhat from one batch to another. Thus, one batch of lesser acute toxicity induced (after 5 months) merely a depression of respiration and lipid biosynthesis from acetate along the whole nerve, as might be expected in the early stages of the lesion. Slices of brain cortex from these same animals showed an even greater depression of the same functions.

In 32 hens, paralysis invariably developed within 2–3 weeks of the first injection. When the food intake was not controlled, the birds lost 33–40 per cent of their weight in 23–27 days. At this time, respiration and lipid biosynthesis from acetate were severely depressed in the sciatic nerves and in selected samples of white matter from the

spinal cord. In birds which were force-fed during the experimental period, the body weight remained almost unchanged; oxygen uptake by the sciatics was still depressed; but in the white matter a significant drop in respiration and lipid biosynthesis from acetate persisted only in one sample corresponding to the zone of most severe damage as indicated by the Marchi stain. Hence, in the later stages of Mipafox paralysis in hens, the effects of nutritional deficiency are superimposed on those of the drug. For this and other reasons, it is pointed out that rats lend themselves better than hens to the study of certain aspects of paralysis induced by organo-phosphorus compounds.

The effects of dietary restriction alone were also studied in hens. After 6–8 days of total food deprivation the birds had lost 13–27 per cent of body weight. In the sciatic nerves, respiration and especially the incorporation of acetate and phosphate into the total lipids were significantly depressed. Samples of white matter from the spinal cord were not demonstrably affected.

The pathogenesis of the peripheral neuropathy induced by organo-phosphorus compounds is discussed. The data here presented do not favour the view that Mipafox is a 'specific myelin poison'; rather, they add circumstantial evidence in favour of a primary central lesion.

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A HISTOCHEMICAL STUDY OF DPN-DIAPHORASE IN HUMAN WHITE MATTER WITH SOME NOTES ON MYELINATION

REINHARD L. FRIEDE
Mental Health Research Institute and Laboratory of Neuropathology,
University of Michigan, Ann Arbor, Michigan, U.S.A.

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THERE is some indication of differences in the anatomical and chemical organization of individual fibre tracts in the brain, such as the outstanding activity of alkaline phosphatase in the retroflex bundle of the guinea pig (SHIMIZU, 1950) and the different rates of synthesis of acetylcholine in various fibre tracts (FELDBERG and VOGT, 1948).

This paper reports on the distribution of DPN-diaphorase in human white matter. The distribution of enzyme activity in human grey matter will not be touched upon. It resembles, in principle, that of succinic dehydrogenase in the guinea pig brain which, in turn, is similar to that of several oxidative enzymes in cat brain as documented recently in an histochemical atlas (FRIEDE, 1961). The activity of oxidative enzymes is much less in white matter than in grey matter, as shown by both biochemical and histochemical data. Histochemical studies revealed detailed differences in the cytological enzyme distribution in the various tracts. Some data on myelination and on certain features of enzyme transport in axons were included in this study because they imply a difference in the chemical organization of the tracts.

MATERIAL AND METHODS

Histochemical techniques. DPN-diaphorase was demonstrated with the histochemical techniques of FARBER, STERNBERG and DUNLAP (1956) and of SCARPELLI, HESS and PEARSE (1958) with equally satisfactory results. Large series were available from six human brains without pathological changes. Brains could be used up to 10 hr after death with perfect histochemical results. The tissue was fixed in formalin (SAMORAJSKI, 1960); identical results were obtained in sections from fresh frozen tissue and from formalin-fixed tissue. Contrary to SAMORAJSKI's observations, an initial increase in enzyme activity was not observed following exposure to formalin; instead the enzyme activity decreased gradually. For fixation, the tissue had to be cut in 5–8 mm slices. In thicker specimens fixation did not extend to the centre of the block which was still pink after one day and yielded a somewhat stronger reaction. The decrease in enzyme activity was greatly delayed by immediate cooling of the tissue in a refrigerator following exposure to formalin; there was little loss in activity during the first 2–3 days. This facilitated handling of much larger series than was possible with fresh frozen sections. Fair results were obtained with tissue stored for 5 days in the refrigerator. After 5 days there was a considerable weakening of the reaction. Temporary removal of the tissue from the refrigerator also weakened the reaction. Fixation in CAJAL's (1928) brom-formalin, as used for glia impregnations, completely destroyed enzyme activity. Sections were cut at 30 μ, washed in distilled water, and incubated in a Technicon water bath at 38° for 2 hr in either of the above media. The volume of incubation medium was adjusted according to the number and size of sections as too much tissue decreased the general staining intensity. About 15 ml of fluid for 10 large sections (20 × 20 mm) taken from a region with strong enzyme activity, was a satisfactory proportion. After completion of the reaction, one half of the material was dehydrated and mounted in balsam and the rest in glycerin–gelatin. Mounting in balsam improved the discernibility of cellular details in white matter. The

findings were controlled by comparison with sections mounted in glycerin–gelatin (PEARSE, 1960). Some sections were routinely counterstained with chromalum–gallocyanin to obtain sharper cytological localization.

Cell counts. During the cutting of tissue for the enzyme reaction, alternate sections were saved for cell counts; they were stained by chromalum–gallocyanin, dehydrated, and mounted in balsam. These sections represented a 30 μ layer of tissue regardless of the shrinkage of the thickness during dehydration. The shrinkage in width was determined by comparison with sections from the same block which were mounted directly in glycerin–gelatin without dehydration. Dehydrated sections were mounted individually and the extent of the shrinkage varied in the range of 5–10 per cent. This shrinkage and its variation have to be taken into consideration when the present data are compared with data on fresh tissue. Volume changes due to fixation in formalin were neglected. The numbers of glial cells in fibre systems were counted with an ocular-net micrometer with 100 squares. All counts were made by the same person. Endothelial cells and the glial septa between the fibre bundles in the brain stem were excluded; the use of thick sections facilitated the identification of endothelial cells since the sections always contained larger segments of the vessels. No differential counts of the types of glial cells (astrocytes, oligodendroglia, microglia, etc.) were made. The above technique was also used to count cells with enzyme activity. At least 20 counts were made for each of the values reported in the text; the data were subjected to statistical analysis.

Densitometric measurements of myelin. Sections were cut from formalin fixed tissue at 30 μ; they were stained for a carefully standardized time in the same batch of Sudan black during one working period. The sections were washed in water so that no stain was dissolved from the tissue as by differentiation in alcohol. Measurements were made on a microscopic screen with a Welch Densichron densitometer. The densitometer readings were calibrated as follows: unstained tissue or tissue subjected to lipid extraction was considered to have a density of zero and stained tissue of normal white matter was arbitrarily chosen to be 100.

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RESULTS

Distribution of DPN-diaphorase in human white matter

A survey of the distribution of DPN-diaphorase activity in the various parts of white matter revealed considerable variations in the number of glial cells with enzyme activity and the intensity of the reaction in them. Enzyme activity was also found in axons. Since the myelin sheaths lacked enzyme activity, the axons were readily discernible within the surrounding tissue. The following terms were defined to describe enzyme activity in axons:

A *prominent* reaction showed a continuous, heavy staining of the axon and a very clear-cut separation of the axon from the adjacent tissue; the sharpness of the reaction resembled silver impregnations.

A *distinct* reaction permitted identification of axons but the staining intensity was either not very heavy or not entirely continuous throughout the length of the fibre.

A *vague* reaction showed weak activity in axons, which was, at times, difficult to distinguish from surrounding tissue.

A *diffuse* reaction showed a homogeneous distribution of formazan crystals in the tissue and localization in axons was not possible. It was felt that the diffuse reaction was localized, at least in part, in processes of glial cells. This was suggested by certain patterns which simulated the arrangement of glial processes, but no definite evidence for this localization was obtained. A 'diffuse' reaction was always associated with large numbers of oligodendroglia.

A quantitative estimate of the enzyme activity in glial cells was made by counting, in sections which were not counterstained, all glial perikarya which could be discerned by a positive reaction. Glial cells indiscernible because of negative reaction were

represented by the difference between the number of perikarya with enzyme reaction and the total number of cells. Both series of counts were done simultaneously under the same conditions on material from the same subjects. In addition, an effort was made to describe the intensity of the reaction in the individual perikarya with the terms weak, medium or strong. One would expect that the number of cells discernible by a positive reaction would depend largely on the general intensity of the reaction but in practice variations in staining intensity had relatively little effect on the counts. The difference between cells with enzyme activity and cells with very little or none, was of sufficient magnitude to establish a histochemical gradient. The intensity of the reaction did not influence its pattern of distribution in axons or glial cells. If the reaction was too weak, both were barely discernible; a stronger reaction improved the contrast without change in the pattern. The material used for this investigation showed equal staining intensity throughout. The data (Table 1) show an inverse relationship between the reaction in axons and the number of glial cells with enzyme activity.

There were striking variations in the numbers of glial cells in the various fibre systems (Table 1), while the number in any tract was relatively constant as shown by counts at various levels of the same tract (pyramidal tracts, No. 18, 19, 20, 24); brachium conjunctivum (No. 21, 22); frontopontine tracts (No. 31, 32). Spot checks made at many levels of other fibre systems gave similar results.

A sizeable portion of the glial cell population contained enzyme activity but there was always another portion with extremely little or no enzyme activity. Most intriguing was the inverse relationship between the enzyme reaction in axons and the number of glial cells with enzyme reaction. Fibre tracts with strong activity in the axons contained few glial cells with enzyme activity (and had a low total glial count); on the other hand, fibre tracts showing no enzyme activity in axons contained many glial cells with enzyme activity (Figs. 1–8).

These patterns were to some extent related to the thickness of the fibres, since only thicker fibres had a 'prominent' reaction. The strong reaction in thick fibres probably resulted from the greater amount of axoplasm in them; one thick (A-) fibre contains as much axoplasm as 28 thin (C-) fibres (diameters taken from LLOYD and CHANG, 1948). A thick axon, therefore, provides for the fibre a much better supply of axoplasm and with it enzyme activity than a thin axon. From these findings it was concluded that both axon and glial cells share the total enzyme activity required for the maintenance of a fibre, but the distribution between axon and glial cells varied in the individual tracts. The availability of axoplasm apparently was one of the factors determining this variance.

Some particular findings emphasized the correlation of axonal and glial enzyme supply as against the assumption that there was merely a relation between the number of glial cells and the number of fibres. The ventral spinal roots (Fig. 1) contained only a negligible number of glial cells with enzyme activity—too few to be of metabolic significance—while all the fibres showed a prominent reaction. These fibres thus had almost exclusively '*axonal*' enzyme supply, regardless of the total number of glial cells available. In the frontal pole (Fig. 8), on the other hand, most of the enzyme activity available was definitely within glial perikarya, even if all of the 'diffuse' reaction was attributed to axons, which was unlikely. Goll's tract (Fig. 2) had relatively high total glial counts; however, only a few cells showed enzyme activity, while all the axons

TABLE 1.—SURVEY OF SOME HUMAN CENTRAL TRACTS

No.	Tract	Cells with enzyme activity	Total glia cells	Reaction in axons	Reaction in glia*
		in thousands per mm³ tissue ± (S.D.)			
1.	Radix ventralis	1·5 (1)	10 (4)	prominent	S
2.	Nervus hypoglossus	<1	<1	prominent	
3.	Nervus facialis	9 (3)	31 (7)	prominent	S
4.	Nervus trochlearis	6 (2)	41 (10)	prominent	S
5.	N. oculomotorius (thick fibres only)	8 (2)	29 (7)	prominent	S
6.	N. oculomotorius (common trunk)	<1	21 (5)	prominent	
7.	Tractus mesencephalici n. trigemini	5 (1)	52 (6)	prominent	M
8.	Nervus trigemini (motor)	9 (2)	27 (6)	prominent	S
9.	Nervus trigemini (common trunk)	24 (5)	57 (11)	mixed	M/S
10.	Fasciculus gracilis (Goll)	10 (3)	89 (10)	prominent	W
11.	Corpus trapezoides	11 (3)	30 (5)	distinct	
12.	Fibrae arcuatae ventrales	12 (4)	51 (8)	distinct-vague	W
13.	Fasc. longitudinalis med. (caudal)	10 (4)	39 (6)	distinct	W/S
14.	Fasc. longitudinalis med. (cranial)	9 (4)	40 (7)	distinct	S
15.	Fasciculus cuneatus (Burdach)	21 (4)	65 (6)	distinct	M
16.	Tractus spinocerebellaris	8 (2)	32 (7)	distinct-prominent	M/S
17.	Corpus restiforme	14 (4)	42 (5)	distinct	M
18.	Tractus pyramidalis (decussatio)	16 (4)	60 (8)	mixed-distinct	S
19.	Tractus pyramidalis (medulla)	18 (4)	55 (11)	mixed-distinct	S
20.	Tractus pyramidalis (peduncles)	17 (5)	48 (11)	mixed-distinct	S
21.	Brachium conjunctivum (proximal)	17 (5)	37 (10)	distinct-vague	S
22.	Brachium conjunctivum (distal)	15 (4)	45 (9)	distinct	M
23.	Thalamus, lamella externa	14 (2)	59 (10)	distinct	M
24.	Tractus pyramidalis (capsula)	20 (5)	54 (10)	distinct	S
25.	Radiatio optica	21 (6)	96 (13)	distinct	M
26.	Fundus nuclei olivaris inferior	32 (4)	71 (8)	diffuse	M
27.	Radix trigemini descendentis	29 (6)	100 (23)	diffuse	W
28.	Subst. medull. cerebelli (central)	27 (5)	61 (7)	mixed	M
29.	Subst. medull. cerebelli (subcort.)	37 (7)	68 (8)	diffuse	M
30.	Tractus pontocerebellaris	28 (5)	64 (8)	diffuse	M
31.	Tractus frontopontinus (pedunculi)	36 (5)	97 (8)	diffuse	M
32.	Tractus frontopontinus (capsula)	42 (7)	91 (10)	diffuse	M
33.	Commissura anterior	83 (12)	141 (11)	diffuse	M
34.	Tractus mammillothalamicus	31 (4)	86 (8)	diffuse	W
35.	Corpus callosum (central)	27 (3)	85 (9)	mixed-distinct	S/M
36.	Subst. alba (frontal pole)	98 (12)	141 (10)	diffuse	M
37.	Subst. alba (temporal pole)	79 (9)	130 (13)	diffuse	W
38.	Subst. alba (parieto-central)	33 (6)	114 (11)	mixed	M
39.	Tractus solitarius	19 (4)	164 (26)	diffuse	W

* Strong (S), medium (M), weak (W)

1–9. Counts in all of the nerves refer exclusively to the intracerebral portion of the roots.

11. Corpus trapezoides: these axons contained very strong activity of succinic dehydrogenase in the guinea pig; in man, their enzyme activity was distinctly weaker but still confined to the axons.

28, 29. Cerebellum: the cerebellar white matter showed a gradient between the subcortical parts and the central parts, as indicated by the difference between the samples 28 and 29. Sample 28 is from the vicinity of the dentate nucleus.

35. Corpus callosum: the data in the corpus callosum varied between the rostrum, corpus, and splenium. These portions showed a composition similar to their respective lobes. Sample 35 was derived from the level of the motor region and showed the lowest counts of all the portions of the corpus callosum.

36–38. The number of cells with enzyme activity in the cerebral hemispheres decreased in the region of the U-fibres, although the total count did not change significantly. All the counts were made in the central parts of the lobes. A decrease of the glial enzyme activity in the neighbourhood of grey matter with strong reaction was also found in the perforating fibre bundles in the striatum.

In the pyramidal tracts, the medial longitudinal fascicle, and many other tracts, the definition of the features of the tract was difficult because of the wide-spread fibre spectrum of these tracts.

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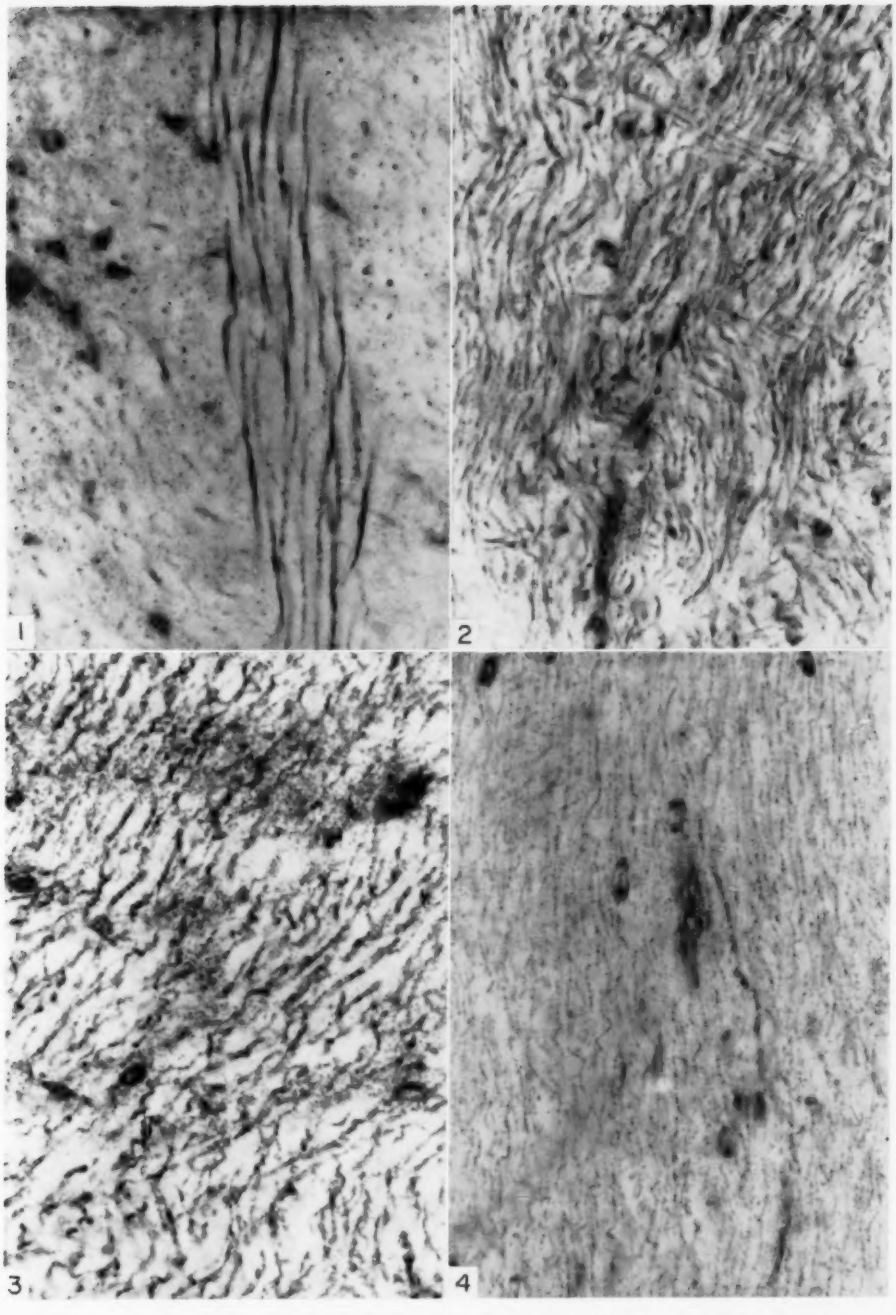

FIGS. 1–8, 14, and 15 show fields of exactly equal magnification (275×) from sections of equal thickness, printed on equal grade papers. No counterstain was used in any of the illustrations which all show the DPN-diaphorase reaction.

FIG. 1.—Anterior roots in the cervical cord, crossing the ventral tracts; note the absence of glial cells and the strong enzyme activity in the axons.

FIG. 2.—Goll's tract near its nucleus in the upper cervical cord.

FIG. 3.—Spinocerebellar tract.

FIG. 4.—Eighth nerve.

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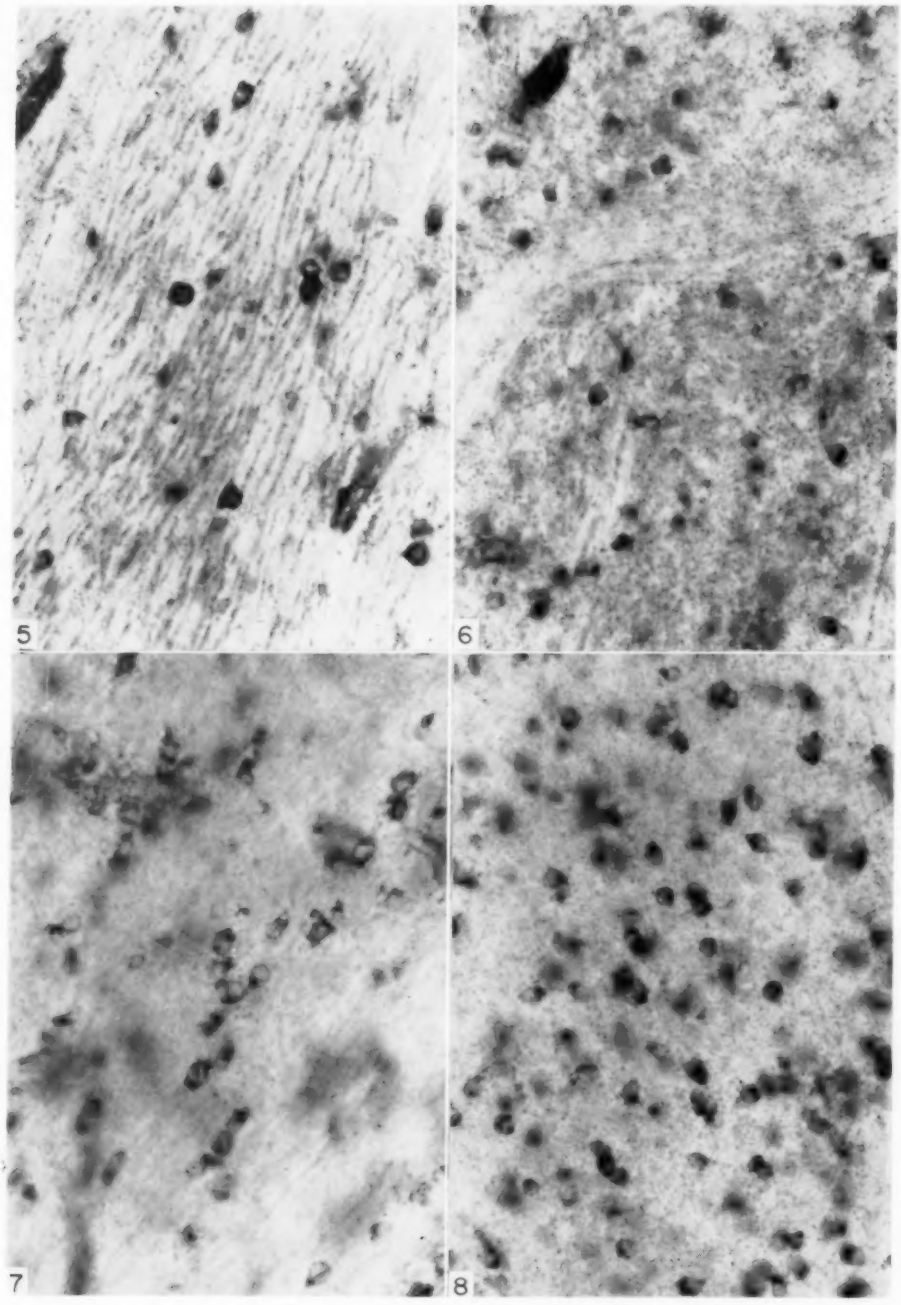

FIGS. 1–8, 14, and 15 show fields of exactly equal magnification (275×) from sections of equal thickness, printed on equal grade papers. No counterstain was used in any of the illustrations which all show the DPN-diaphorase reaction.

FIG. 5.—Burdach's tract, adjacent to Goll's tract. Figs. 2, 3, and 5 were taken from the same section. Note the weaker reaction in the axons and the increase of glia cells with enzyme activity in Fig. 5.

FIG. 6.—Olivary hilus. Absence of glial cells with enzyme activity in the transfixing fibre bundle in the upper part of the picture; this bundle contains fibres with 'axonal' activity (slightly out of focus).

FIG. 7.—Pontocerebellar fibres.

FIG. 8.—Frontal pole, central white matter; note the scarcity of activity outside of glial perikarya, as compared with Figs. 1, 2, and 3.

exhibited a 'prominent' reaction. These observations emphasize the inverse relationship of the enzyme supply of axons and glial perikarya found throughout this material.

Since these are considerable gradations of oxidative enzyme activity among nuclei of the brain, these might represent another factor modifying the activity in the fibre tracts. The scarcity of cells with enzyme activity among the glial cells in the solitary tract was explained by the fact that the nucleus of the solitary tract is one of those with the weakest activity of oxidative enzymes among all the centres of the brain.

Orientation studies showed that the distribution of succinic dehydrogenase in human white matter resembled that of DPN-diaphorase. The histochemical techniques for cytochrome oxidase did not permit sufficient distinction of cytological details in white matter, but this enzyme is known to have a distribution similar to succinic dehydrogenase. It is likely that the pattern described for DPN-diaphorase is similar to the distribution of other oxidative enzymes.

Histochemical distinction of types of glial cells

Glial cells with enzyme activity were probably oligodendroglia, as suggested by a comparison of their distribution and number. HORTEGA (1928) distinguished four types of oligodendroglia by the mode of branching of their processes. Cells of type IV resemble Schwann cells, are bipolar or fusiform with few processes, and are attached to, or encompass, medium or thick nerve fibres. This type showed strong enzyme activity in the cytoplasm which was sharply delineated from the encompassed myelin sheath (Figs. 10, 13). Cells of type III are large and have one or two processes which form cytoplasmic rings or spirals around thicker axons. These cells, their processes, and the rings were clearly identified by a strong reaction (Figs. 11, 12, 13) and were seen best in transverse sections of fibre tracts. The reaction in the rings was so strong that rings could be mistaken for glial perikarya and represented a source of error in the counts. Such rings were found only in tracts where the glial perikarya showed strong enzyme activity; if a ring was connected to a perikaryon it was always a perikaryon with strong enzyme activity. Glial cells with strong enzyme activity thus represented mostly oligodendroglia of types III or IV. These cells were distributed characteristically in certain tracts (Table 1) in agreement with HORTEGA's findings.

Oligodendroglia of types I and II are characterized by many long and delicate processes; these two types were not distinguished from each other histochemically. On the basis of their frequency, cells with medium or weak activity in the perikarya probably represented these types. These cells (Figs. 7, 8) predominated in the white matter of the cerebral and cerebellar hemispheres. Their long processes probably contained some of the enzyme activity of the diffuse reaction.

The number of cells without enzyme activity obviously included the population of astrocytes (POPE, 1952, counted 26 per cent astrocytes in the rat's cerebral white matter). The dense marginal layer of astrocytes at the outer surface of the brain almost completely lacks DPN-diaphorase activity but pathological (swollen) astrocytes contain considerably more enzyme activity than normal ones (FRIEDE, 1958).

The problems of the histochemical specificity of certain types of glia were spotlighted by some observations made in fish species (northern pike, *Esox lucius;* and bowfin, *Amia calva*). The ependymal cells of lower species have long processes which extend to the surface of the brain where they form small pedicles—'Tanycyten' (HORSTMANN, 1954). These ependymal cells, with their processes and pedicles, contained

strong activity of DPN-diaphorase (Fig. 9) and were, in certain regions, the tissue element with maximal activity. Some of the functions of glial cells might be performed by this primitive ependyma, since these species had relatively few glia cells: Medulla oblongata (numbers in thousands per mm^3) total cells, 29(6); cells with enzyme, 12(3). This supports WLASSAK's (1898) concept, which attributed to these cells some significance for the formation of myelin in lower species.

Myelination of human white matter

The data reported in this paragraph indicate that the normal enzyme patterns described above determine certain features of myelination. Morphological changes and an increase in the number of glial cells during myelination were described by BOLL (1874), HORTEGA (1928), BELLONI (1930), MORRISON (1931), LINELL and TOM (1931), PENFIELD (1924) and ALPERS and HAYMAKER (1934). ROBACK and SCHERER (1935) emphasized the increase in the number of glial cells during myelination (Myelinisationsgliose) which was confirmed by counts in the chick embryo by BENSTED *et al.* (1957). Only DEKABAN (1956) denied an increase in oligodendroglia during myelination (dorsal spinal tracts of the rabbit). HILD (1957) on the other hand, observed the formation of myelin about axons in tissue cultures without any morphological participation of glial cells. The axoplasm, however, showed an accumulation of mitochondria at the site of formation of myelin.

There is some histochemical evidence of increased activity of the glia during myelination. KOENIG (1958) reported an increased turnover of proteins. The activity of cytochrome oxidase was found to be increased (MARINESCO, 1924). It was reported by FRIEDE (1959*a*) that in the rat's brain, succinic dehydrogenase increased and decreased in the glia during myelination within a period of about 6–8 days; the onset of this increase preceded the visible formation of myelin. Increased activity was found from the fifth day on in the lateral fibre tracts of the medulla oblongata. The activity increased at about the tenth or twelfth day and reached a maximum at about the fourteenth day in the spinal trigeminal tract, the pyramidal tract, the corpus restiforme, parts of the dorsal and lateral fibre tracts of the cervical spinal cord, the central white matter of the cerebellum and cerebrum, and the fibre stratum of the cornu ammonis. The activity decreased in most fibre systems after the sixteenth day, but it was more persistent in the pyramidal tract, parts of the dorsal tracts of the cervical medulla, the middle cerebellar peduncle, the cornu ammonis, and parts of the cerebral white matter, where activity was found up to the 20th day.

The myelination of the human brain was studied in three regions at eight phases of development. Unfortunately, histochemical material was obtained for only one of these phases. Cell counts were made as described above, the cell numbers were compared with the progress of myelination; densitometric measurements of the staining intensity of the myelin with Sudan black was used as a crude and chemically unspecific parameter of the increase of lipids in the tissue. As a means of control, additional measurements were made in each specimen in regions of grey matter which lacked myelinated fibres, such as the upper layers of the cerebral cortex or the putamen. BRANTE's (1949,) data on lipid content were recalculated for wet weight which approximated the per volume approach in tissue sections. A comparison is made (Fig. 16) of these data with the densitometric measurements; the latter evidently provided a usable parameter of myelination. A comparison of the individual curves reveals that changes

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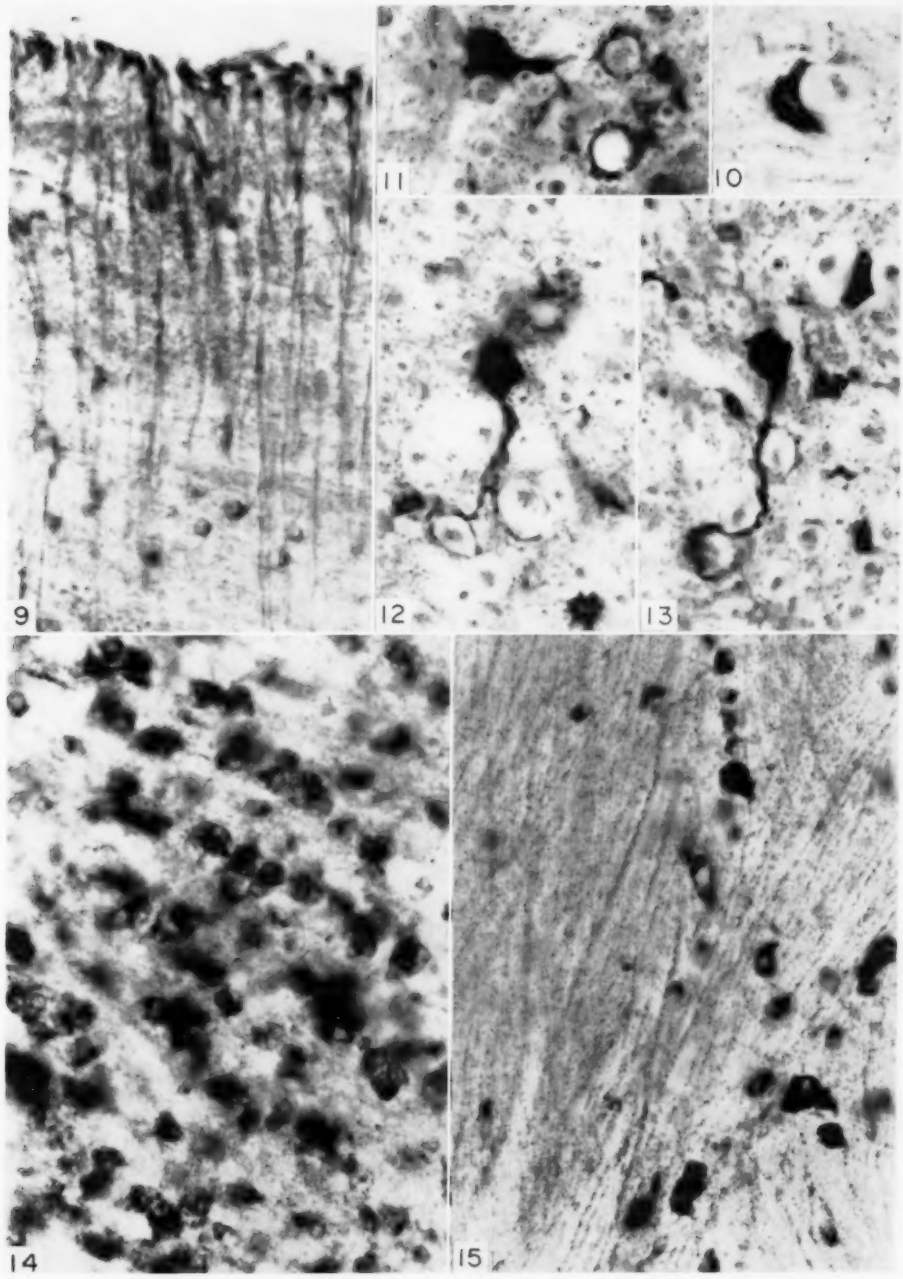

FIGS. 1–8, 14, and 15 show fields of exactly equal magnification (275×) from sections of equal thickness, printed on equal grade papers. No counterstain was used in any of the illustrations which all show the DPN-diaphorase reaction.

FIG. 9.—Processes of ependymal cells with DPN-diaphorase activity in the brain of the bowfin (275×).

FIG. 10.—Oligodendroglia, type IV, encompassing a thick fibre; another such cell is seen in the upper part of Fig. 13 (440×).

FIGS. 11–13.—Oligodendroglia, type III, as identified by its typical formation of rings or spirals around thick axons (440×).

FIGS. 14 and 15.—Pyramidal tract in the capsula interna; the pictures show homologous regions in the newborn (Fig. 14) and adult (Fig. 15).

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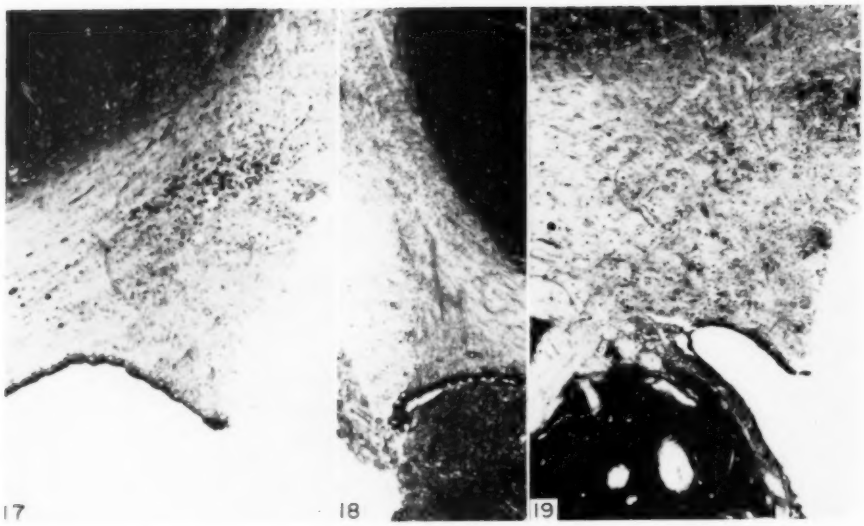

FIGS. 17–19.—Transections of various fibre systems in the cerebral hemispheres of the guinea pig. Note the differences among tracts of the intensity of damming of DPN-diaphorase activity; *e.g.*, in Fig. 17 in the upper and lower part of the picture. Little damming is found in a thin-fibred portion of the corpus callosum (Fig. 18). The dammed fibres represent only a small fraction of the total fibre population (Fig. 19). The dark regions represent the cerebral cortex and the nucl. caudatus (32×).

in the lipid content of the white matter of the hemispheres result from a superposition of several, specific changes in individual fibre systems.

Table 2 and Fig. 16 represent the course of myelination in the pyramidal tracts (internal capsule), and in the white matter of the parietal and the temporal lobes. All three regions showed an increase of glial cells which distinctly preceded the formation of lipids (BENSTED *et al.*, 1957). This increase occurred first in the pyramidal tracts,

TABLE 2.—GLIA COUNTS DURING MYELINATION OF HUMAN FIBRE TRACTS

Age	Pyramidal tract (capsula interna)	Parietal lobe	Temporal lobe
	(Glial cells in thousands per $mm^3 \pm$ S.D.)		
6 foetal months	24 ± 6	34 ± 9	79 ± 12
8 foetal months	136 ± 15	65 ± 8	64 ± 8
Newborn	155 ± 10	73 ± 10	62 ± 5
5 months	124 ± 10	118 ± 10	60 ± 8
10 months	93 ± 10	106 ± 8	120 ± 9
15 months	81 ± 9	123 ± 11	124 ± 13
4 years	67 ± 7	114 ± 14	124 ± 13
Adult	54 ± 10	114 ± 11	129 ± 12

then in the parietal white matter, and last in the temporal white matter, in accordance with the time course of myelination (Fig. 16). The proliferation of cells was accompanied by changes of the nuclei, which led to the differentiation of oligodendroglia (ROBACK and SCHERER, 1935). Following myelination, however, conspicuous differences were found among the regions. In the parietal and temporal lobes, the proliferation of glial cells increased to and maintained the level found in adults. The pyramidal tracts, in contrast, showed a peak in cell count during myelination, followed by a continuous decrease to the low level found in adults. These data provide evidence of the striking differences in the features of myelination among fibre systems.

Histochemical data were available only for the newborn; at this phase, myelination had not started in the parietal and temporal lobes, and there were few cells with enzyme activity. The pyramidal tract was at the peak of myelination and showed many cells with strong DPN-diaphorase activity, exceeding every adult fibre system (Table 3). Figure 14 shows this impressive increase in enzyme activity as compared to the appearance of the homologous region in adults (Fig. 15). In the newborn, no axons could be discerned by enzyme activity, and there was very little 'diffuse' reaction.

The most logical interpretation of these findings was that the glia of the pyramidal tract proliferated because the axons did not have their normal enzyme supply as yet. As the axons matured and attained their normal enzyme content (Fig. 15), the participation of the glia lost significance. The different features of myelination thus seem to reflect the chemical organization of fibre tracts in adults. One would predict that the myelination of other tracts with 'axonal' enzyme supply would follow the pattern of the pyramidal tracts. Orientation counts were made in the intracerebral rootlets of the hypoglossal and (motor) trigeminal nerve of a human fœtus (3 1/2 months) at the very outset of myelination. Myelination starts in the motor roots of human cranial nerves at about 14 weeks of age (KEENE and HEWER, 1931) and only a few

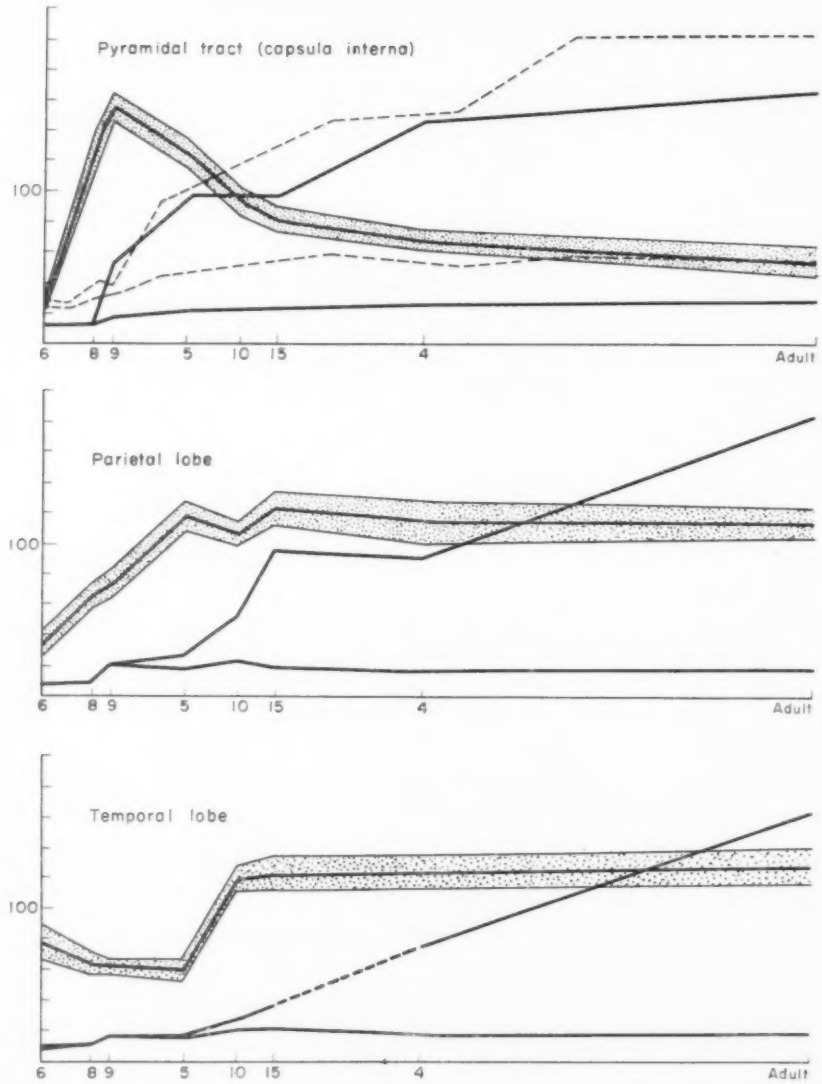

FIG. 16.—Glial cell numbers (Table 3) and myelination in the human brain. The dotted curve shows the numbers of glial cells in thousands per mm³ tissue (± S.D.) The solid lines show the densitometric data of myelin formation (upper part of the curve) and of grey matter without myelinated fibres (lower part of the curve). The interrupted lines show BRANTE's (1949) data on total lipids, recalculated for wet weight; BRANTE's curve may be considered as superposition of the three densitometric curves.

The age scale was computed as a logarithmic function from conception (unit one year); this method puts data concerning growth in a more proper relationship.

The relatively high cellularity of the temporal lobe at the sixth month results from the migration of neuroblasts through white matter and has no meaning as to the local cell population.

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fibres with newly formed sheaths were found in the above material. The cell numbers were, in thousands per mm^3: hypoglossal nerve, 136; trigeminal nerve, 148; and pyramidal tracts, 20. The pyramidal tracts were counted as a control to show that the high numbers of cells in the roots were not due to a higher cellularity of the foetal brain. The myelination of the nerve roots, thus, clearly followed the pattern of the pyramidal tract, which adds further support to the concept that the normal chemical

TABLE 3.—ENZYME CHANGES DURING MYELINATION IN THE HUMAN NEWBORN

	Cells with enzymic activity		Total glial cells	
	Newborn	Adult	Newborn	Adult
	(in thousands per mm^3 tissue ± S.D.)			
Pyramidal tracts (capsula)	108 ± 11	20 ± 5	155 ± 10	54 ± 10
Parietal	13 ± 3	33 ± 6	74 ± 10	114 ± 11
Temporal	17 ± 3	79 ± 9	62 ± 5	130 ± 13

organization of the fibres determines the features of myelination. No correction was made for growth in these data, since the increase of glial cells occurred within a short period, during which the growth of the brain was negligible.

The significance of transport of axoplasm in various types of nerve fibres

Oxidative enzymes, particularly DPN-diaphorase, in the axons derive from the perikaryon and are transported distally in the axon (FRIEDE, 1959*b*). The gradations of DPN-diaphorase activity in axons implied that transport mechanisms were not of equal significance in all fibre systems. The enzyme supply of the anterior roots, for example, depended almost exclusively on transport in axons, while fibres in the frontal pole relied on the local glia which contained the major share of the total enzyme supply of this region. Transportation might even represent a cause rather than a consequence of the various enzyme patterns in white matter, since the resistance to flow through a tube increases inversely to the square of the diameter.

These conclusions were examined in silver impregnations (HORTEGA's, silver carbonate technique (1928)) of axons in seven specimens made available by courtesy of Dr. K. SCHARENBERG; these were biopsy material from surgical resections, diagnostic punctures and chordotomies obtained 1–5 days after surgery. The material was selected to show: (1) a sharply delineated interruption of tracts, (2) a cut longitudinal to the course of the fibres, and (3) no other pathological changes.

Only a fraction of the total number of fibres exhibited a typical damming of axoplasm; the percentages of swollen fibres and of thick fibres were of the same order. The intensity of damming generally decreased with the diameter of the fibres. This relationship was noted by CAJAL (1928): "the extent of retrograde propagation" (now generally recognized as damming, WEISS and HISCOE, 1949) "is proportional to the thickness of the medullated fibre."

Additional experiments in which the white matter in the cerebral hemispheres

was transected in guinea pigs showed that the fibre swellings seen in silver preparations represented a damming of axoplasm with DPN-diaphorase activity. The animals were killed after 2–4 days. Histochemical preparations showed the typical damming of enzyme activity in the swollen fibre stumps as has been studied in detail in a previous investigation (FRIEDE, 1959*c*). In accordance with the human material (Table 4) only a fraction of the fibre population showed damming of enzyme activity (Fig. 19). The extent of damming varied considerably among tracts. Some portions of the corpus callosum showed very little damming (Fig. 18), and the damming was confined to certain tracts in other parts of white matter (Fig. 17).

TABLE 4.—FIBRE COUNTS IN SURGICALLY TRANSECTED HUMAN WHITE MATTER

Type	Age (years)	Survival (days)	Total count*	% Thick fibres	% Swollen fibres†
Puncture hemisphere	45	1	143	22	20
Puncture hemisphere	39	5	139	42	55
Puncture hemisphere	52	2	116	17	29
Puncture hemisphere	28	2	106	10	21
Puncture hemisphere	64	2	101	14	18
Puncture hemisphere	15	4	110	16	56
Chordotomy	42	2	476	26	31

* Counts of the total no. of axons crossing a line of given length, rectangular to the course of the fibres. Counts made about 10 mm from site of interruption.
† Percentage of terminal swellings found in a strip of tissue containing the entire length of fibres between the original count and the site of interruption.

DISCUSSION

The histochemical distribution of DPN-diaphorase in white matter revealed an inverse relationship of the enzyme activity found in axons and in glial cells. Tracts with very prominent reaction in axons showed few, or no, glial cells with enzyme activity. On the other hand, tracts in which axons were not discernible by a positive reaction, showed many glial cells with enzyme activity. The conclusion was drawn from these findings that axons and glia shared the total enzyme activity of a given tract to a widely variable degree among tracts. The tracts with 'axonal' enzyme activity always contained thick fibres, while tracts with 'glial' enzyme activity contained thin fibres. The availability of axoplasm with enzyme activity, thus, was one of the factors determining the activity in glial cells. Axons and glia evidently formed a symbiotic unit; they were "mutually serviceable, and there was established between them something like a symbiosis comparable to the well-known symbiosis of fungi and algae." (CAJAL, 1928, p. 459.)

There was some indication that the pattern of DPN-diaphorase reflected the general pattern of tissue oxidation, since succinic dehydrogenase showed a similar distribution. The patterns of the individual oxidative enzymes in brain tissue generally resemble each other (FRIEDE, 1961).

The number of glial cells per unit volume was found to vary considerably among fibre tracts, but it was rather constant along the course of a given tract. The values obtained by cell counts were of the same order as the data obtained by measuring DNA (ELLIOTT and HELLER, 1957; ROBINS, SMITH and EYDT, 1956). The data from

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cell counts should be somewhat lower than those from DNA determinations, as endothelial nuclei and contamination by adjacent tissue were eliminated in the counts. Preference was given to cell counts in this investigation as many of the tracts counted were too minute to permit a clean macroscopical dissection.

Most of the cells with DPN-diaphorase activity apparently represent oligodendroglia, while normal astrocytes seem to contain little oxidative enzyme activity (LOWRY *et al.*, 1954; POPE *et al.*, 1956; ROBINS and SMITH, 1953). The strong enzyme reaction is in keeping with the appearance of oligodendroglia in the electron microscope (LUSE, 1956; FARQUHAR and HARTMANN, 1957; DEMPSEY and LUSE, 1958), the high oxidative metabolism of oligodendrogliomas (VICTOR and WOLFF, 1937; ELLIOTT and HELLER, 1957) metabolic studies (POPE and HESS, 1957; HYDÉN and PIGON, 1960; HESS, 1961) and the greater vulnerability of oligodendroglia. Histochemistry even enables one to distinguish certain types of oligodendroglia as described by HORTEGA (1928); these types are distinguished by gradations of the enzyme reaction in their perikarya.

The normal patterns of enzyme distribution in fibre tracts were related to certain features of myelination. The histochemical patterns of the adult white matter of the parietal and temporal lobes were very similar to each other; both showed predominance of enzyme activity in glial cells. During myelination, both showed an increase in the number of glial cells, which levelled into the permanent adult numbers.

The adult pyramidal tracts and cranial nerve roots were characterized by predominant enzyme reaction in axons and by few glial cells with enzyme activity. During myelination, the number of glial cells in these tracts increased, but, in contrast to the other two regions, decreased after myelination. No enzyme activity was discernible in axons at the peak of myelination. The glial proliferation was considered compensatory and the decrease of glial cells after myelination appeared to be a consequence of the later increase of axon enzyme supply. The type of glial reaction during myelination, therefore, was determined by the type of chemical organization of the adult fibres.

One might speculate on the possibility of myelination without interaction of glia under extreme conditions and the capability of the axon to increase significantly its enzyme activity. HILD (1957) observed, in tissue cultures, increased numbers of mitochondria in the axoplasm at the site of myelination; there was apparently no relation of glial cells to this type of myelination. An increase of the axonal diameter during myelination (SPEIDEL, 1933; DEKABAN, 1956) also indicates some significance of the axon.

DEKABAN (1956) denied an increase in oligodendroglia during myelination; this statement was correct, in as much as the increase of the cell numbers was not simultaneous with, but preceded the formation of myelin, as shown by BENSTED *et al.* (1957) and in this study. DEKABAN's data, as a matter of fact, show the increase of cells exactly where it is expected to be, but only the terminal phase of the increase was counted. It is logical that the multiplication of cells precedes myelination since not the cell division, but the metabolism of the cells thus produced is important for the formation of myelin.

The relative distribution of DPN-diaphorase in axon and glial perikarya suggested that transportation of axoplasma with DPN-diaphorase activity was not of equal significance in all fibre systems. The oxidative enzymes found in the axoplasm

evidently are derived from the perikaryon and are being carried distally in the axoplasm (for further literature see FRIEDE, 1959*b*). This conclusion was in keeping with the observation that the extent of damming of axonal flow in transected fibres varied considerably with the fibre's diameter; thin fibres showed very little damming, or none at all. The extent of damming of DPN-diaphorase activity in experimentally transected tracts likewise varied among tracts. If there are differences in transportation and damming in transected fibres, the enzyme changes in the neurons proper might vary as well. KLEIN (1960), accordingly, found an increase of succinic dehydrogenase in spinal ganglion cells following sciatic transection, while this enzyme decreased in motor cells (FRIEDE, 1959*b*).

In conclusion, axonal and glial cells represent a symbiosis in which the share of metabolic activity of either axons or glia depends on several factors such as the availability of axoplasm, (thickness of fibre), difficulties of axonal enzyme transport, functional activity, and metabolic demands of the sheath (myelination). These findings do not permit the drawing of any conclusions as to the final role played by the glia in the morphological formation of the sheath. Moreover, they apply specifically to central white matter; they should not be applied indiscriminately to Schwann cells in peripheral nerves, since the histochemical architecture of peripheral nerves differs considerably from that of central tracts.

The selection of DPN-diaphorase for this investigation was not determined by speculations as to its possible biochemical significance in lipid metabolism but rather by the fact that this technique was sufficiently sensitive and reliable to demonstrate consistently minute gradations of enzyme activity in white matter. It is known, nevertheless, that the biosynthesis of phosphatides requires the presence of tissue oxidation and that inhibition of the latter inhibits the formation of phosphatides (ARTOM and CORNATZER, 1951; TAUROG, CHAIKOFF and PERLMAN, 1942; SCHACHNER, FRIES and CHAIKOFF, 1942). Changes in the activity of oxidative enzymes, thus, may indirectly indicate changes of more specific phases of lipid biosynthesis. The persistence of high glial counts in certain fibre systems after completion of myelination agrees with the persistent high rate of turnover of certain lipids during and after myelination (WAELSCH, SPERRY and STOYANOFF, 1940).

SUMMARY

A systematic study of the histochemical distribution of DPN-diaphorase in human white matter showed an inverse relationship of the enzyme activity in axons and in glial cells. A strong reaction in axons was accompanied by few glial cells with enzyme activity; tracts in which axons could not be discerned by a positive reaction, contained many glial perikarya with enzyme activity. One of the factors determining this inverse relationship was the thickness of the fibres (availability of axoplasm with enzyme); thick fibres always showed an 'axonal' enzyme supply and thin fibres a 'glial' enzyme supply.

The glial perikarya with enzyme activity represented mostly oligodendroglia. Certain types of oligodendroglia could be distinguished histochemically by gradients of the reaction in the perikarya.

The normal histochemical organization of fibre tracts was found to determine certain features of myelination. Preceding the formation of myelin, there was a sharp increase in the number of glial cells and an increase of enzyme activity in them. The

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population of glia after completion of myelination differed among tracts; high glial numbers were maintained in tracts with 'glial' enzyme supply, while the number of cells decreased in tracts with 'axonal' enzyme supply.

Another factor influencing the histochemical architecture of a fibre tract was the increasing difficulty of axonal flow and transport of enzymes in thinner fibres. This was demonstrated by differences in the intensities of damming of axonal flow and transport of enzyme activity after transection of mixed fibre populations.

The central nerve fibres represent a complex symbiosis of axons and glial cells, in which the metabolic share of each depends on several factors, such as availability of axoplasm (diameter of the axon), intensity of axonal flow, functional activity, and metabolic demands of the sheath (myelination).

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OXIDATION OF TRICARBOXYLIC ACID CYCLE INTERMEDIATES BY NERVE CELL BODIES AND GLIAL CELLS*

ANDERS HAMBERGER
Department of Histology, University of Gothenburg, Gothenburg, Sweden

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THE COMPLEX anatomy of the nervous system makes a study of small functional units desirable and it is self-evident that quantitative analyses of isolated nerve cells and glia offer a hope of elucidating many aspects of the relationship between these two main components of the central nervous system. Different results have been obtained in macrochemical investigations of the relative importance of nerve cells and glia. MCILWAIN (1955), POPE (1958), and KOREY and ORCHEN (1959) ascribe less activity to the glia than to the nerve cells whereas LOWRY *et al.* (1954) and ELLIOTT (1959) report the glia to be more active metabolically. During the last few years interest in the role of glia in the metabolism of the nervous system has been renewed, largely because of the sensitive analytical methods which have become available. Two different approaches have been used. LOWRY *et al.* (1956, 1957*a*,*b*) made colorimetric and fluorimetric enzyme analyses on frozen-dried material, while HYDÉN and co-workers (1958, 1960) measured enzymes manometrically and determined the nucleoprotein content on fresh microdissected material; both procedures seem to have their specific advantages.

The method of HYDÉN has been employed in the present investigation with the aim of detecting characteristics with regard to the oxidation of tricarboxylic acid cycle intermediates by the two structural components, neurones and glia in the lateral vestibular nucleus of Deiters.

MATERIAL AND METHODS

Albino rabbits of both sexes, weighing 1·6–2 kg were used. They were made unconscious by an air embolus and the carotid arteries were cut to let the remaining heart beats drain the blood from the body. The brain was removed, a 1–2 mm section was cut from the desired part and placed in sterile 0·25 M-sucrose solution, slightly coloured with methylene blue to facilitate visualization of the nerve cells. The giant cells of the lateral vestibular nucleus of Deiters, called the Deiters' nerve cells (BRODAL and POMPEIANO, 1957) were studied. The section was placed on a paraffin plate under a stereomicroscope with hand supports and examined under 64 or 100 times magnification. A piece of stainless steel wire, 20 or 30 μ thick, mounted on a drawn glass tube, was used for freehand dissection of single nerve cells together with adhering glia, mainly oligodendrocytes (HYDÉN and PIGON, 1960). All samples were placed in a small drop of the reaction mixture in an agar-coated glass cup. By gentle manipulation the nerve cells were freed from the clump of surrounding glia. The nerve cells remained relatively undamaged, with most of the dendrites attached. The clump of glial cells was trimmed to get a volume approximately equal to that of the nerve cell. In this manner a number of nerve cell bodies and glial clumps are collected in the substrate drop.

Identical values are obtained for the weight per unit volume of fresh, and of frozen and dried nerve cell bodies and glial cells from the Deiters nucleus (HYDÉN, 1958), so that the results as expressed in this

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paper on the basis of volume give the same relation as would be obtained on a dry weight basis. Oxygen consumption was measured by the manometric method of ZEUTHEN (1953) and ZAJICEK and ZEUTHEN (1956). The detailed use of this method in the study of nerve cells has been described by HYDÉN, LØVTRUP and PIGON (1958). The samples were aspirated with 0·3–0·5 μl of the reaction medium into microdivers which were made from Pyrex glass capillaries. They were about 15 mm long, weighed 0·2–0·6 mg, and had an air volume of 0·05–0·1 μl. The filled divers were sealed with melted wax, adjusted to buoyancy at atmospheric pressure and transferred to flotation vessels in a thermostatically controlled water bath. The divers were left for 30 min for temperature equilibration, manometric readings were then taken every 10–12 min for about 2 hr. Four divers were run simultaneously. Oxygen consumption was calculated according to the method of ZEUTHEN (1953) and results were expressed in 10^{-4} μl O_2 per hr per nerve cell or per corresponding volume of glia. The reaction medium used was modified after GALLAGHER, JUDAH and REES (1956). Standard medium: 6·7 mM-$MgSO_4$; 25 mM-KCl; 13·3 mM-sodium phosphate buffer (pH 7·4); 0·02 mM-cytochrome *c* (Sigma); mM-ATP (Schwarz); 0·175 M-sucrose. To this was added one of the following substrates: 13 mM-sodium pyruvate + 0·5 mM-Na L-malate; 13 mM-Na-α-oxoglutarate; 15 mM-Na-L-glutamate (commercially available). The concentrations given are those in the final mixture in the diver.

In order to absorb CO_2 the flotation medium was made up of 0·1 N-NaOH. All measurements were carried out at 37°.

TABLE I.—OXYGEN CONSUMPTION OF NERVE CELL BODIES AND GLIAL CELLS OF DEITERS' NUCLEUS FROM THE RABBIT

Expressed in 10^{-4} μl O_2/hr. Mean net values ± S.E.M.*

Substrate	Nerve cell body	Glia	*P*	N/G
Pyruvate + malate	0·8 ± 0·1 *n* = 6	1·9 ± 0·47 *n* = 12	<0·01	0·4
α-Oxoglutarate	1·1 ± 0·15 *n* = 13	0·9 ± 0·19 *n* = 14	—	1·2
Glutamate	2·2 ± 0·34 *n* = 21	0·6 ± 0·14 *n* = 11	<0·01	3·7

* Values corrected for endogenous respiration
n = number of measurements
P = the probability value for a significant difference between nerve cell body and glia values
N/G = Nerve cell body/Glia

RESULTS

The results from the oxidation of pyruvate + malate, α-oxoglutarate and glutamate are shown in Table 1. A few experiments were made with glucose and with succinate as substrates. Values for glucose differed very little from those for the endogenous respiration (Table 2). Succinate showed a tendency to give high values; a ratio between nerve cell body and glia (N/G) of about 0·5 was obtained, in agreement with the results for succinoxidase in the same cells (HYDÉN and PIGON, 1960).

Oxidation of pyruvate may be similar to that of succinate in that the greatest activity was found in glia (N/G of 0·4 and 0·5 respectively). The nerve cells on the contrary seem to be especially equipped for oxidation of glutamate (N/G, 3·7). Results given by many authors (MCILWAIN, 1955; TOWER, 1960*a*,*b*; WAELSCH, 1960) show that glutamate can be oxidized through a number of different pathways, some of which feed into the tricarboxylic acid cycle. α-Oxoglutarate has an intermediate position, being oxidized almost equally well by glia and nerve cells (N/G, 1·2).

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The values for endogenous respiration, i.e. the oxygen consumption in the standard medium without added substrate (Table 2), approached zero for the nerve cells and were slightly higher for glia. The mean values in Table 2 were subtracted from the original mean values for substrate oxidation in the reaction media in an attempt to get the characteristic N/G for the different substrates. As the values in Table 2 are relatively small this correction has little effect on the ratios. It should be stressed that endogenous respiration in the presence of substrates might be even less. The autoxidation of the substrates was too low to be measured.

TABLE 2.—ENDOGENOUS RESPIRATION OF NERVE CELL BODIES AND GLIAL CELLS OF DEITERS' NUCLEUS FROM THE RABBIT
Expressed in 10^{-4} μl O_2/hr. Mean values ± S.E.M.

Nerve cell body	Glia	*P*	N/G
0·04 ± 0·03 *n* = 9	0·3 ± 0·06 *n* = 7	<0·01	0·1

n = number of measurements
P = probability value for a significant difference between nerve cell body and glia values
N/G = Nerve cell body/Glia

DISCUSSION

As pointed out by WAELSCH (1960) the determination of the relative respiration of neuronal and non-neuronal elements in bulk isolations is uncertain. In the macroscale approach to the problem, the separation of brain tissue into nerve cells and glia is impossible. Astro- and oligodendroglia tumours offer a possibility of obtaining material for macroscale measurements on a pure sample, although the tumours are not composed of the same cells as those which surround a nerve cell. The use of isolated nerve cells and equally small amounts of glia affords a morphologically sound basis for the N/G determinations. One inevitable objection is that the metabolism of different kinds of glia can hardly be equated and that the N/G from one part of the brain does not reflect the relations in the whole nervous system. In the survey below, the present results are compared with those on glial cells reported in the literature. It should be stressed, however, that the author's results are obtained on isolated oligodendrocytes immediately surrounding a nerve cell. The admixture of astrocytes does not exceed 10 per cent (HYDÉN and PIGON, 1960).

The values obtained by MCILWAIN (1955) on the respiration of brain slices in an unfortified glucose saline medium give a respiratory ratio for grey to white matter of about 2. BRIERLY and MCILWAIN (1956) conclude that neoplasms of glial origin have a considerably lower respiratory rate than subcortical white matter. The replacement of glucose by succinate did not increase the oxygen consumption of an oligodendroglioma, as might have been expected from the present values. When glutamate was added to the unfortified medium, the respiratory rate of slices of normal grey matter increased by 100 per cent, while that of subcortical white matter was completely unaffected. This observation is in good agreement with the present findings on N/G for glutamate which was the least effective substrate of those tested here to support glial oxygen consumption. BRIERLY and MCILWAIN (1956) discuss the results of HELLER and ELLIOTT (1955) which show a high respiratory rate in glial tumours.

HELLER and ELLIOTT suggest that glial cells respire more actively than *some* nerve cells, a conclusion not contradicted by the present findings. KOREY, ORCHEN and BROTZ (1958) found that the greatest respiratory activity in a glia preparation was achieved when pyruvate and succinate were used in combination. It was necessary to add glutamate as well to achieve maximal respiration in grey matter.

The measurements of ROBINS, SMITH and EYDT (1956) of different enzymes in architectonic layers of motor and visual cortex, show a certain parallelism between nerve cell density and tricarboxylic acid cycle enzymes. This correlation did not hold equally well for glutamic dehydrogenase. Yet in view of the present experiments one would expect a distinct correlation if this enzyme were concerned in glutamate oxidation. However, the complex anatomical structure in this part of the brain does not permit a sharp differentiation of neuronal and glial layers.

LOWRY (1956, 1957*a*,*b*) in analyses of single spinal ganglion cells and their glia capsules found N/G values, on a dry weight basis, of about 2 for glutamic dehydrogenase and of 6 for glutamic-aspartic transaminase. The corresponding ratio in this work for oxidation of glutamate (N/G, 3·7) lies midway between these values. LOWRY associates the two mentioned enzymes with hexokinase and malic dehydrogenase in a group of enzymes which show relatively high activity in nerve cells. LOWE, ROBINS and EYERMAN (1958) found an almost negligible activity for glutamic decarboxylase in white matter as compared with grey matter. Although the results are variable, they emphasize that glutamate metabolism is rather specific for nerve cells, an observation supported by some positive clinical results in treatment of epilepsy and mental deficiency with glutamate (WAELSCH and PUTNAM, 1943; TOWER, 1960). The enzymes of the tricarboxylic acid cycle are of special interest in the study of carbohydrate metabolism. LOWRY's values show an N/G value of 1 for isocitric dehydrogenase. HYDÉN *et al.* (1958) and HYDÉN and PIGON (1960) gave N/G values on a volume basis for spinal ganglion cells of about 0·15 for succinoxidase and 0·5 for cytochrome oxidase; for Deiters' cells the values were 0·5 and 0·4 respectively. From these observations and from the values in Table 1 it is concluded that some of the steps in the tricarboxylic acid cycle are most effectively performed in the surrounding glia, while others predominate in the nerve cells.

Measurements with mitochondrial preparations from whole brain give the total respiratory capacity of neuronal and glial mitochondria but variable results have been obtained by different authors because of variation in the preparation of the mitochondrial fraction. BRODY and BAIN (1952) found that pyruvate, of the substrates tested here, gave the highest values for oxygen consumption. For α-oxoglutarate and glutamate lower values were found, and succinate gave an even lower value. LØVTRUP (personal communication) obtained the following respiratory relationship for brain mitochondria: pyruvate, 3; α-oxoglutarate, 1; glutamate, 1·5; succinate, 3·5. The figures presented in this work on the oxidation of the same substrates give an N/G value of about 0·5 for pyruvate and succinate; for the two other substrates the N/G exceeded 1·0. These results indicate a certain trend; when glia in the microdiver experiment show the highest activity, the oxidative capacity of the mitochondrial preparation is large, and *vice versa*.

The N/G for one enzyme can be changed by increased functional demand on the nervous system (HYDÉN 1959, 1960) indicating that there exists a functional relationship between nerve cell and glia at the biochemical level. The position of glia in

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relation to other structures of the nervous system and the special functional demand that follows from this, probably are the most important factors which influence its metabolic activity.

SUMMARY

The oxidation of tricarboxylic acid cycle intermediates was measured. Manometric microdeterminations of oxygen consumption were made on isolated nerve cells and surrounding oligodendroglia from Deiters' nucleus of rabbit. The nerve cell bodies mainly utilize glutamate, whereas succinate and pyruvate are essentially oxidized by the glia. α-Oxoglutarate is used equally by the two components.

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THE INHIBITORY ACTION OF 3-HYDROXYTYRAMINE, GAMMA-AMINOBUTYRIC ACID (GABA) AND SOME OTHER COMPOUNDS TOWARDS THE CRAYFISH STRETCH RECEPTOR NEURON*

E. G. McGeer, P. L. McGeer and H. McLennan
Kinsmen Laboratory of Neurological Research, and Department of Physiology, University of British Columbia, Vancouver, Canada

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The concept of inhibitory processes occurring in the nervous system of various organisms is part of classical neurophysiology, but the idea of inhibitory neurons with axons whose endings release a specific inhibitory transmitter substance is relatively new (Eccles, 1957). Evidence for the presence of inhibitory cells in the central nervous system of mammals is now abundant but, as yet, no inhibitory transmitter has been chemically identified. Curtis and Watkins (1960*a*) have, however, suggested some criteria that such a substance should fulfil. An inhibitory transmitter might be contained in Factor I, a crude preparation from mammalian brain described originally by Florey (1954) and Florey and McLennan (1955*a*, *b*), and which has been shown by McLennan (1960) to meet some of the criteria set up by Curtis and Watkins (1960*a*, *b*).

Identification of inhibitory transmitter substances is an important objective of current neurophysiological research, and suitable bioassay procedures are essential for such work. One convenient and widely used preparation is the slowly-adapting stretch receptor neuron of the crayfish. Inhibition of the discharge of this neuron was used by Bazemore, Elliott and Florey (1957) to identify GABA as an active component of Factor I preparations. This and subsequent work (Elliott, 1958) led to the suggestion that GABA might account for most or all of the activity of Factor I-containing brain extracts. However, it was shown later that not all of the actions of Factor I are duplicated by GABA (McLennan, 1957*a*). Moreover, a number of other amino acids, some of which occur in mammalian brain, have also been shown to have inhibitory effects on the crayfish stretch receptor neuron (Bazemore, Elliott and Florey, 1957; Edwards and Kuffler, 1959). The effects of GABA have since been investigated on many biological systems (Roberts, 1960), and the concept is developing that this amino acid functions, at least in the central nervous system of mammals, not as a specific inhibitory transmitter substance, but as a neuronal depressant (Curtis, Phillis and Watkins, 1959).

It now appears that the crayfish stretch receptor neuron is not a specific bioassay system; such lack of specificity may be of advantage in indicating the inhibitory characteristics of compounds not previously suspected of possessing such properties. In the study now reported, a series of compounds has been tested for inhibitory

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activity on the crayfish stretch receptor neuron in an attempt to define as closely as possible the nature of the inhibitory receptor sites.

Most compounds previously shown to be inhibitory towards the crayfish preparation (KUFFLER and EDWARDS, 1957; BAZEMORE, ELLIOTT and FLOREY, 1957; EDWARDS and KUFFLER, 1959) are compounds containing an acidic and a suitably separated basic function. The majority have been aliphatic amino acids, but there is no reason to believe that other substances possessing such general characteristics might not also be active. The principles which guided the selection of compounds for this study were the presence of acidic and basic functions, and the separation and spatial orientation of these functional groups.

METHODS

Most of the samples were high grade commercial preparations. Each material was chromatographed in at least three solvents (*n*-butanol/acetic acid/water (4:1:1), *iso*propanol/ammonium hydroxide/water (8:1:1), and methanol/butanol/benzene/water (2:1:1:1)) and shown to have the expected R_F and to be free of major contamination. Extensive chromatographic data on most of the aromatic materials used in this study have been published elsewhere (MCGEER, ROBERTSON and MCGEER, 1961). The four materials with unexpectedly high activities (imidazoleacetic acid hydrochloride, DL-noradrenaline hydrochloride, L-adrenaline, and 3-hydroxytyramine hydrochloride) were each recrystallized and assayed in comparison with unrecrystallized material. Except in the case of 3-hydroxytyramine, recrystallization did not significantly alter the observed activity. In the case of 3-hydroxytyramine, the activity was materially increased as discussed later. Tyramine (*o* and *m*) and 2:5-dihydroxyphenylethylamine were made from the corresponding amino acids by incubation with a decarboxylase prepared from *Str. faecalis* (MITOMA *et al.*, 1957). The tyramine derivatives were extracted into butanol (UDENFRIEND, WEISSBACH and CLARK, 1955), separated on Whatman no. 1 paper by chromatography with an *n*-butanol/acetic acid/water (4:1:1) solvent system, and eluted from the paper with 'crayfish saline' (VAN HARREVELD, 1936). The quantity in the eluant was estimated by spectrophotofluorometric comparison with the parent amino acid. Table 1 lists the chromatographic and spectrophotofluorometric data on these compounds, which were not prepared in sufficient

TABLE 1.—CHROMATOGRAPHIC AND SPECTROPHOTOFLUORIMETRIC CHARACTERISTICS OF *o*- AND *m*-TYRAMINE AND OF 2,5-DIHYDROXYPHENYLETHYLAMINE

	o-Tyramine	*m*-Tyramine	2,5-Dihydroxyphenylethylamine
R_F in BuOH/AcOH/H_2O (4:1:1)	0·58–0·66	0·55–0·62	0·36–0·40
Colour with diazotized sulphanilic acid	yellow	yellow	yellow-pink
Activation peak at pH 6·6	270	270	290
Fluorescent peak at pH 6·6	319	319	330

quantities to allow crystallization. The substances to be tested were made up in aqueous solution at a suitable concentration (usually 1–10 mg/ml) just before testing and were then further diluted at least 1 in 10 with 'crayfish saline'. The pH was adjusted where necessary to 6·5–7. Each solution was given a code number so that the operator performing the bioassay was unfamiliar with the identity. Each substance was tested at least twice on different days and with different crayfish. Materials showing activity were tested on at least three different days.

Pacifastacus leniusculus (Dana) was the species of crayfish used. The stretch receptor organ in one of the abdominal segments was dissected free of overlying tissue so that the nerve cell body was exposed. Solutions to be tested were run over the exposed cell. After 15 sec exposure, excess fluid in the shell of the crayfish was sucked away, and the effect upon the rate of discharge of the neuron noted. The solution was then washed off thoroughly with 'crayfish saline'. If a solution caused complete blockage of the neuronal discharge, it was diluted and tested until the threshold

TABLE 2.—ACTIVITY OF HOMOLOGUES AND SIMPLY MODIFIED ANALOGUES OF GABA ON THE STRETCH RECEPTOR NEURON OF THE CRAYFISH

Feature under consideration	Compound	Activity found*	Reference compound	As per cent of activity of reference compound†	Activity reported by EDWARDS and KUFFLER (1959). As per cent of GABA activity
(a) Chain length	glycine	Inactive (<0·1 unit)	GABA	nil	5%
	β-alanine	1·8, 6·2, 1·8 (av. 3·3)	GABA	10%, 12%, 12% (av. 11%)	5%
	GABA	82, 62, 21, 52, 52, 52, 155, 72, 103, 206, 41, 82, 21, 15, 206, 103, 41, 103, 26 (av. 80)			
	5-amino-*n*-valeric acid	1·3, 1·3, 3·5 (av. 2)	GABA	3%, 3%, 5% (av. 4%)	8%
	6-amino-*n*-caproic acid	1·3, 1·3, 0·1 (av. 1)	GABA	2·5%, 3%, 0·2% (av. 2%)	1%
	7-amino-*n*-caprylic acid	Inactive (<0·13)	GABA	nil	
(b) Branching or substitution	DL-β-amino-*n*-butyric acid	0·27, <0·1, 0·6 (av. 0·3)	GABA	0·7%, <0·2%, 0·05% (av. 0·2%)	0·2%
			β-alanine	10%	
	DL-4-amino-3-hydroxy-butyric acid	5·9, 8·9, 8·9 (av. 8)	GABA	17%, 12%, 14% (av. 14%)	
(c) Simple changes in acid function (with or without substitution)	taurine	0·25, 0·12, 0·6 (av. 0·3)	GABA	0·6%, 0·2%, 0·3% (av. 0·4%)	0·5%
			β-alanine	10%	
	γ-aminobutyrylcholine‡	1·6, 3·8, <2·2, <2·2	GABA	4%, 2%, <4%, <3%	
(d) Simple changes in basic function (with or without substitution)	guanidinoacetic acid	12, 13, 35 (av. 23)	GABA	43%, 19%, 110% (av. 57%)	58%
			Glycine	>23,000%	
	β-guanidinopropionic acid	13, 98, 65, 26, 16, 393, 26, 52, 17 (av. 79)	GABA	21%, 440%, 125%, 63%, 100%, 190%, 25%, 130%, 17% (av. 123%)	72%
			β-alanine	2400%	
	L-2-amino-3-guanidino-propionic acid	14·6, 7, 5 (av. 9)	GABA	35%, 9%, 32% (av. 25%)	
			β-alanine	275%	
	γ-guanidinobutyric acid	2·2, <1·4, <1·4, 1·4, 0·6	GABA	3·5%, <3%, <7%, 3·5%, 0·8%	12·5%

Compounds of this type which proved non-inhibitory in repeated tests at 1 mg/ml were: glycine, 7-aminocaprylic acid, DL-2:4-diaminobutyric acid, glycylglycine, ethanolamine-O-phosphate, γ-butyrobetaine.HCl, L-2-amino-3-ureidopropionic acid and canavanine sulphate. 11-Aminoundecanoic acid, ethanolamine-*o*-sulphate, betaine.HCl, creatine, 3-mercaptopropionic acid, and carnitine.HCl, tested at 100 μg/ml. were inactive.

* Units of activity defined as the reciprocal of the concentration, in m-moles per l., which produces a threshold block when applied to neuron.

† Percentage of GABA activity is referred to same preparation since GABA was tested on each crayfish; percentage of other reference compounds is calculated on average figures.

‡ Slight activity shown by this material may be due to traces of free GABA formed by hydrolysis; such traces of GABA are sometimes visible in chromatograms of this substance.

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concentration was reached. The threshold concentration was considered to be that which would cause a transient (about 20 sec) blockage of the discharge. The method is essentially that described by FLOREY (1954) and by ELLIOTT and FLOREY (1956).

When the effects of blocking agents were to be determined, the test solutions to be assayed were measured and then the stretch receptor organ was soaked for a period of 10 min with the blocking agent. Excess blocking agent was sucked away, and test solutions were assayed in the usual fashion to determine new threshold concentrations. Each time the stretch receptor organ was washed with 'crayfish saline', however, fresh blocking agent was applied before a new solution was tested. In practice it was difficult to wash the blocking agent off once applied.

RESULTS

The compounds tested fell into several distinct chemical classes, and the relative inhibitory activities in each group are listed below in separate tables. The activity of any compound is expressed in units; these are defined as the reciprocal of the concentration, in millimoles per litre, which produces a threshold block when applied to the stretch receptor neuron. The individual assay results for each active material are presented in Tables 2–4, except for those obtained with one particular crayfish. These are presented separately in Table 5, because this crayfish proved so unusually sensitive to 3-hydroxytyramine and some other aromatic materials that it was felt inclusion of the results with those obtained on other crayfish would give unrealistic averages for the few aromatic substances tested on that particular crayfish. The other nineteen crayfish used for this study varied in their sensitivity, with the variability tending to be greater with more active substances such as 3-hydroxytyramine and less with weaker substances such as GABA. However, the data on these other crayfish could be satisfactorily grouped.

Non-inhibitory compounds were not tested at concentrations higher than approximately 1–5 μmole per ml to avoid nonspecific effects; compounds reported as inactive might show slight inhibitory activity at higher concentrations.

Only inhibitory activity has been considered here. A number of the non-inhibitory materials caused excitation and a few caused muscle contraction; these actions are not reported.

Effects of chain length, branching and substitution

A series of straight chain, aliphatic amino acids were first tested (Table 2*a*). These data, in good agreement with those previously reported (EDWARDS and KUFFLER, 1959), show that GABA is by far the most active inhibitor in this series, and confirm that the three carbon spacing between functions is optimal or near optimal for inhibitory activity.

The relative activities of aliphatic amino acids in which the carbon chain is branched or substituted are shown in Table 2*b*. All such compounds which we have been able to test are much less active than the corresponding straight chain, unsubstituted, aliphatic amino acid; such a diminution in inhibitory activity on substitution or branching has been well explored (KUFFLER and EDWARDS, 1957; EDWARDS and KUFFLER, 1959).

Effects of changing acidic or basic function

Substitution of the carboxylic acid group by a sulphonic acid, a choline ester, an O-phosphate, or an O-sulphate function results in serious diminution or loss of

TABLE 3.—INHIBITORY ACTIVITY TOWARDS THE CRAYFISH STRETCH RECEPTOR NEURON OF SOME SELECTED IMIDAZOLES AND OTHER NITROGEN HETEROCYCLES

Substance	Activity found	
	In units	As per cent of GABA activity on the same preparation
Imidazoleacetic acid.HCl	56, 40, 113, 81, 202, 65, 162, 16 245, 29, 58, 88, 51 (av. 93)	92%, 50%, 550%, 158%, 100%, 158%, 195%, 100% 120%, 28%, 140%, 85%, 196% (av. 152%)
1-Methylimidazole-4-acetic acid	<1·8, <1·8, 1·8, <1·8, <1·8	<4%, <4%, 3·5%, <3·5%, <2%
Imidazolepropionic acid*	2·5, 0·7 (av. 1·6)	2·4%, 2·7% (av. 2·5%)

* Only two assays done.

Substances of this class tested repeatedly and found to be non-inhibitory at the concentrations used were:

At 1 mg/ml	At 100 μg/ml	
Urocanic acid	Imidazolepyruvic acid	3-Indoleacetic acid
Histidine. 2HCl†	1-Methylimidazole-5-acetic acid	5-Hydroxyindole-3-acetic acid
Tryptamine. 2HCl†	4-Hydroxymethylimidazole. HCl	Adrenchrome
	Histamine. 2HCl	2:3-Dihydroxyindole
At 0·5 mg/ml	*N*-Acetyl-L-histidine	Pyridoxamine. 2HCl†
	Histidinol. 2HCl	5-Hydroxytryptamine
3-Indolepropionic acid†	L-Ergothionine. $HCl \cdot H_2O$	Hydantoin-5-acetic acid
	L-2-Thiolhistidine.HCl	Nicotinic acid
	Indican sulphate	DL-Pipecolic acid
	8-Hydroxyquinoline	L-Proline

† Showed some activity on an unusually sensitive crayfish (see Table 5) but was completely inactive on three other preparations.

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TABLE 4.—INHIBITORY ACTIVITY TOWARDS THE CRAYFISH STRETCH RECEPTOR NEURON OF SOME PHENOLIC AMINES AND OTHER BENZENOID DERIVATIVES

Substance	In units	As per cent of activity of Grade A 3-hydroxytyramine on same preparation	Ratio of activity to that of GABA on same preparation
3-Hydroxytyramine.HCl (Grade A Calif. Biochem. Res. Corp., recrystallized)	15,100, 18,900, 2835, 4720 (av. 10,400)	178%, 200%, 150% (av. 176%)	66, 184, 69, 182 (av. 125)
3-Hydroxytyramine.HCl. (Grade A Calif. Biochem. Res. Corp.)	566, 2830, 755, 3780, 2830, 1890, 1890, 945, 14,200, 1890, 1510, 8500, 9450, 1890, 16,100 (av. 4600)		9, 135, 15, 74, 19, 18, 9, 23, 170, 90, 100, 37, 92, 46, 156 (av. 66)
DL-Noradrenaline.HCl	123, 1030, 1030, 51, 720, 1030, 41, 102 (av. 515)	3%, 54%, 54%, 54%, 5%, 11%, 2% (av. 26%)	2·5, 10, 5, 1, 9, 10, 1, 4 (av. 5·3)
L-Noradrenaline bitartrate.H_2O	75, 468, 75, 28, <70, 17 (av. 110)	0·2%, 25%, 8%, 3%, <0·5%, 1% (av. 6%)	1, 2·5, 1·5, 0·7, <0·9, 0·4 (av. 1·0)
L-Adrenaline bitartrate	55, 65, 185, 18, <4·5, 3 (av. 54)	0·1%, 7%, 10%, 2%, <0·01%, 0·06% (av. 3%)	0·75, 1·25, 0·7, 0·45, <0·1, 0·1 (av. 0·5)
DL-3-Methoxynoradrenaline.HCl	<2·2, <0·22, 0·1, 0·02	<0·2%, <0·02%, 0·001%, 0·0003%	<0·04, <0·005, 0·001, 0·0001
3:4-Dihydroxyphenylacetic acid	<1·6, <1·6, <1·6, 8, <0·2	<0·2%, <0·1%, <0·1%, 0·4%, <0·005%	<0·03, <0·03, <0·008, 0·03, <0·003
DL-3:4-Dihydroxyphenyl-propanolamine.HCl	<2·2, 87, 22, <0·4	—, 9%, 1%, <0·005%	<0·05, 1·7, 0·1, <0·005

Substances of this type which showed no sign of inhibitory activity in two or three tests were: (a) at 100 μg per ml: tyramine. HCl; methylaminoacetocatechol; α-methyl-β-(3:4-dihydroxyphenyl)alanine; 3:4-dimethoxyphenylethylamine; *o*-catechol; 2:5-dihydroxyphenylalanine; *o*-tyrosine; *m*-tyrosine; *m*-aminotyrosine; *m*-aminobenzoic acid; and *p*-aminobenzoic acid. (b) at concentrations indicated: L- and D-isuprel bitartrate.$2H_2O$ (each at 1 mg/ml on two preparations); 2:5-dihydroxyphenylethylamine (27, 27 and 20 μg/ml); *o*-tyramine (43 and 24 μg/ml); *m*-tyramine (50 and 8·4 μg/ml), and 3-methoxy-4-hydroxyphenylethylamine (0·5 and 0·5 mg/ml). The *m*-tyramine did show some activity on one preparation which was omitted from the table above because of its unusual sensitivity (see Table 5).

inhibitory activity. (Table 2*c*). Substitution of a guanidine group for the primary amine group gives a series of guanidino acids which are as active as the amino acids but, since β-guanidinopropionic acid is more active than γ-guanidinobutyric acid or guanidinoacetic acid, the optimal chain length is shifted from three carbon atoms separating the acidic and basic functions to either two or four atoms (depending on which nitrogen of the guanidino groups is acting as the basic function). A guanidino

TABLE 5.—INHIBITORY ACTIVITY FOUND FOR VARIOUS COMPOUNDS ON AN UNUSUALLY SENSITIVE CRAYFISH STRETCH RECEPTOR NEURON

Substance	Activity in Units*
Tryptamine.HCl	2·0
3-Hydroxytyramine.HCl	41,580†
DL-3-Methoxynoradrenaline.HCl	2·2
L-Histidine.2HCl	9·1
3:4-Dihydroxyphenylacetic acid	252
DL-3:4-Dihydroxyphenyl-propanolamine.HCl	1650
m-Tyramine	3·3
GABA	72
Pyridoxamine.diHCl	2·4

Compounds which were non-inhibitory at 100 μg/ml were: L-proline; α-methyl-β-(3:4-dihydroxyphenyl)-alanine; 5-hydroxyindole-3-acetic acid; 2:3-dihydroxyindole; L-isuprel bitartrate. 2:5-Dihydroxyphenylethylamine and *o*-tyramine were inactive at 40 μg/ml.

* Units of activity defined as the reciprocal of the concentration, in m-moles per l., which produces a threshold block when applied to neuron.

† Checked on three separate solutions.

derivative of some interest is L-2-amino-3-guanidinopropionic acid which is 10–30 per cent as active as GABA; this is the first α-amino acid which has, to our knowledge, been found to inhibit the crayfish stretch receptor neuron. Most α-amino acids have been found by others to be inactive (*e.g.* glycine) or excitatory (*e.g.* glutamic acid). The amino group may interact with a terminal nitrogen of the guanidino group and thus be prevented from blocking approach of the carboxyl group to the receptor sites. The corresponding ureido derivative, L-2-amino-3-ureidopropionic acid, was inactive.

Betaines and N-methylguanidinoacetic acid (creatine) are not inhibitors; this indicates that substituted nitrogen atoms are not generally active.

Ring-containing compounds

The results obtained with guanidinopropionic and guanidinoacetic acids suggested that guanidino and amidino derivatives might be more active than simple amino acids if there were appropriate spacing between the acidic and basic functions. Imidazole-acetic acid hydrochloride, which may be considered an amidine derivative with three carbons between the acidic and basic functions, proved more active than GABA on most crayfish preparations. A number of other imidazoles, indoles and other nitrogen heterocycles failed to show any activity except for imidazolepropionic acid which was weakly active and 1-methylimidazole-4-acetic acid which was weakly active in one trial out of five (Table 3). Three others in this category showed traces of activity on

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the one extremely sensitive crayfish preparation (Table 5), but could not be considered active in typical crayfish preparations.

Substituting a stronger acid function for the carboxylic acid group causes diminution or loss of inhibitory activity (Table 2*c*); weaker acids with appropriate structural characteristics can be found among the phenolic and catecholic amines. Results of tests with such compounds are shown in Table 4. An inhibitory activity many times that of GABA was found for recrystallized 3-hydroxytyramine (on average 10,400 *vs.* 80). Such high activity was not shared by the monohydroxyphenylethylamines and no activity was observed in the 3-O-methyl derivatives: DL-Noradrenaline showed an activity comparable to that of GABA but far lower than that of 3-hydroxytyramine. This comparison between 3-hydroxytyramine and noradrenaline is somewhat analogous to that between GABA and DL-β-hydroxy GABA (Table 2*b*) where substituting an hydroxyl group on the aliphatic chain reduced the activity.

The discovery of a high order of activity for 3-hydroxytyramine hydrochloride and of measurable activity for noradrenaline and adrenaline was surprising in view of the previous report by ELLIOTT and FLOREY (1956) that L-adrenaline bitartrate and L-noradrenaline bitartrate were inactive, and particular attention was, therefore, given to the purity of these catecholamines.

The commercial Grade A 3-hydroxytyramine hydrochloride from California Biochemical Research Corporation was tested both analytically and spectrally by the manufacturer. Chromatography in *n*-butanol/acetic acid/water (4:1:1) and methanol/butanol/benzene/water (2:1:1:1) gave no evidence of gross contamination, but a trace of impurity, positive to ultraviolet light and negative to diazotized sulphanilic acid, appeared, which may have been formed during chromatography. Chromatography in basic solvents such as *iso*propanol/ammonia/water (8:1:1) leads to obvious decomposition. The 3-hydroxytyramine hydrochloride was recrystallized from ethanol–ether. Although the recrystallized and unrecrystallized samples gave identical chromatographic and spectrophotofluorometric data, the recrystallized sample proved more than twice as active when tested in parallel on several crayfish preparations (Table 4). Further recrystallization caused no further change in activity. It therefore appears that high purity is essential for reliable assays of 3-hydroxytyramine hydrochloride on the crayfish neuron since even traces of a contaminant may partially block the inhibitory activity of the catecholamine. Elution from a paper chromatogram of the area corresponding to 3-hydroxytyramine in R_F and in colour reactions on parallel chromatograms gave a sample with a very high inhibitory activity consistent with the amount of 3-hydroxytyramine chromatographed. No activity was found in eluants of other areas of the paper including the area showing a trace of the ultraviolet positive material. Partial oxidation of 3-hydroxytyramine caused partial loss of activity but did not appear to produce any blocking agent.

Recrystallization of DL-noradrenaline hydrochloride and of L-adrenaline as the hydrochloride from ethanol/ether did not measurably alter the activity of either.

Compounds such as *m*-aminobenzoic acid, in which the amine function was attached to a benzene ring and the acid function was carboxylic, were inactive even though the acidic and basic functions were separated by an appropriate number of carbon atoms. An explanation based on differences in basicity between aliphatic and aromatic amines seems unlikely in view of the results with aliphatic guanidino acids. These results on aromatic acids emphasize the necessity of considering the relative

orientation of the acidic and basic groups as well as their spacing. All the compounds found to have inhibitory activity could be oriented so that the acidic and basic functions were in approximately the same plane, were separated by about 4 Å, and had no groups interpolated between them. This is illustrated for GABA, imidazoleacetic acid, 3-hydroxytyramine, and β-guanidinopropionic acid in Fig. 1. Compounds such as 2-amino-3-guanidinopropionic acid, which may seem apparent exceptions, are capable of such orientation; this particular derivative may be stabilized in such configuration by chelation between the amino and guanidino groups. *m*-Aminobenzoic acid (also shown in Fig. 1) has the same number of carbons between the amine and carboxyl groups as does GABA, but all possible orientations leave the benzene ring interposed between the prosthetic groups. Conjugation of the carboxyl group with the ring may also be the reason for loss of activity; this possible, though less likely, explanation could be tested by assay of compounds such as 2-aminomethylbenzoic acid.

The difference in activity between DL-noradrenaline and L-noradrenaline bitartrate could not be due to the bitartrate moiety since identical results were obtained with solutions of L-adrenaline bitartrate and L-adrenaline on three separate crayfish preparations. Contamination of the DL-noradrenaline with a small amount of 3-hydroxytyramine is a possibility, but no change in activity was produced by recrystallizing the DL-noradrenaline hydrochloride from ethanol-ether. It seems probable, therefore, that the D-noradrenaline is more active than the L-form, a difference which would be attributable to the spatial orientation of the hydroxyl group, and which suggests that the L-oriented hydroxyl may interfere with approach of the other groups to the receptor.

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Effect of blocking agents

The enormous difference in activity between 3-hydroxytyramine and GABA raised the question as to whether or not these two substances could be acting at the same receptor site on the neuron. To test the possibility that different sites might be involved, the effect of four blocking agents (chlorpromazine, dibenzylene, picrotoxin, and RP-7843) were tried against GABA, 3-hydroxytyramine, imidazoleacetic acid, β-guanidinopropionic acid, and a crude preparation of Factor I made by the method of Florey and McLennan (1955*b*). Picrotoxin has been reported to block the inhibitory effects of GABA and Factor I on this preparation (Elliott and Florey, 1956), while chlorpromazine and dibenzylene prevent the action of catecholamines on many physiological systems (Table 6).

Picrotoxin (mM), blocked about 60 per cent of the activity of GABA, and about 85 per cent of the activity of Factor I, imidazoleacetic acid, and β-guanidinopropionic acid. It was only weakly active against 3-hydroxytyramine. Chlorpromazine (0·15 mM), and dibenzylene, (0·8 mM), on the other hand, almost completely blocked the effects of 3-hydroxytyramine, Factor I, β-guanidinopropionic acid, and imidazoleacetic acid, but were completely inactive against GABA. 3-Dimethylsulphamido-10-(N-methylpiperazinylpropyl)phenothiazine (RP-7843, a powerful new phenothiazine derivative), at 0·15 mM, blocked more than 98 per cent of the inhibitory activity of 3-hydroxytyramine, 70–87 per cent of that of Factor I, and $\leq$ 20 per cent of that of GABA. It is difficult to interpret such results in terms of a single type of receptor site on the stretch receptor neuron.

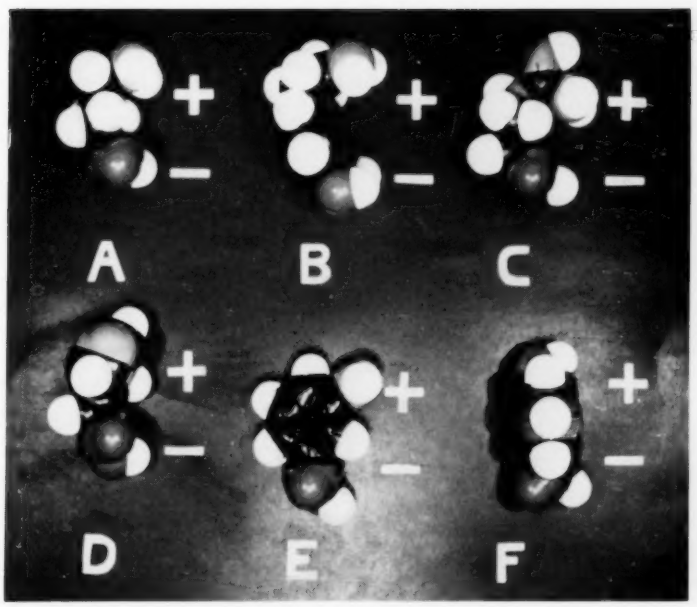

FIG. 1.—Molecular models of some compounds which have been tested for inhibitory activity on the crayfish stretch receptor neuron. A = GABA; B = 3-hydroxytyramine; C = β-guanidinopropionic acid; D = imidazoleacetic acid; and E and F = *m*-aminobenzoic acid in two different orientations. The + and − signs are fixed at 4 Å apart and the molecules in each case are oriented so as to bring an acidic (carboxyl in A, C, D, E and F, and phenolic in B) group in juxtaposition to the − sign, and a basic (amino in A, B, E and F, guanidino $—NH_2$ in C and imidazole —NH— in D) group in juxtaposition to the + sign. *m*-Aminobenzoic acid cannot be satisfactorily oriented. The models are not presented in the ionized form which would exist in solution.

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TABLE 6.—EFFICACY OF CHLORPROMAZINE, DIBENZYLENE, PICROTOXIN, AND RP-7843 AS BLOCKERS OF THE INHIBITORY ACTIVITY OF SELECTED SUBSTANCES ON THE CRAYFISH STRETCH RECEPTOR NEURON

Inhibitor	Percentage of activity lost after pretreatment with:—			
	Picrotoxin (mM)	Chlorpromazine (0·15mM)	Dibenzylene (0·8 mM)	RP-7843(0·15 mM)
3-Hydroxytyramine.HCl	0%, 0%, 50%, 33%	98%, 95%, 95%, 80%, 98%, 93%	80%, 85%	>98%, >99%
Factor I	>75%, >85%†	75%, 58%, 60%	>75%,* >85%†	87·5%, 70%
β-Guanidinopropionic acid	58%, 75%, 87%, 87%	88%, 50%	75%, 75%	<2·5%
Imidazoleacetic acid	75%, 95%, 95%, >87%	75%, 75%	100%, 80%	43%
GABA	73%, 50%, 60%, 50%, 50%	0%, 0%, 0%, 0%, 0%	0%, 0%	20%, ≤20%

* Blocked at a dilution of 1/4000 before dibenzylene; strong excitation noted at 1/1000 after dibenzylene.
† Blocked at a dilution of 1/1500 before picrotoxin or dibenzylene; strong excitation noted at 1/250 after picrotoxin or dibenzylene.

DISCUSSION

The great variability in response of different crayfish preparations to the substances tested is worth comment. This variability was not due to lack of precision in the assay since it was repeatedly shown that a given substance tested under two code numbers on a given day gave concordant results. There was some indication that the more active the substance as an inhibitor, the greater was the variability in response of different preparations. Thus, GABA with an average activity of 80 units showed a 14-fold variation; imidazoleacetic acid with an average activity of 93 units showed a 15-fold variation; and Grade A 3-hydroxytyramine with an average activity of 4600 units

TABLE 7.—ACTIVITY OF SELECTED INHIBITORS TOWARDS SOME INDIVIDUAL CRAYFISH PREPARATIONS

	C	L	N	P	Q	R	S
	(Activity in units†)						
Factor I*	6280	1200	4000	150	600	800	300
Imidazoleacetic acid	113	202	162	16	245	29	58
β-Guanidinopropionic acid	98	—	—	16	393	26	52
GABA	21	206	82	15	206	103	41
3-Hydroxytyramine	2830	1890	14,200	1512	7560	9450	1890

* A molecular weight of 200 for active component(s) was assumed.
† Activity expressed in units defined as the reciprocal of the concentration, in m-moles per l., which produces a threshold block when applied to the stretch receptor neuron.

showed a 73-fold variation. The relative activities of different active chemical classes, *i.e.*, imidazoles, guanidino derivatives, catecholamines, and omega-amino acids, were not consistent from one crayfish preparation to another, and thus no substance was found suitable as a reference standard. The general lack of parallelism is evident in the comparative figures given in Tables 2 to 4 and is illustrated also in Table 7. The activities of imidazoleacetic and β-guanidinopropionic acids did seem to parallel one another, and homologues of GABA followed the response to GABA.

Our data on GABA and closely related compounds (Table 2) agree remarkably well with those obtained by EDWARDS and KUFFLER (1959) using crayfish of species *Orconectes virilis* and *Procambares alleni*. We found the guanidino acids to be comparable in activity to the ω-amino acids, while EDWARDS and KUFFLER found them slightly less potent, but the difference was small. By contrast, however, we have found the catecholamines to be highly active, whereas ELLIOTT and FLOREY (1956) previously reported that L-adrenaline bitartrate and L-noradrenaline bitartrate were inactive at a concentration of 1 mg/ml (less than 0·3 units in each case) on stretch receptor neurons of *O. virilis*.

Almost all of the compounds found by others or by us to be inhibitory contain both acidic and basic functions with appropriate spacing between them. There are a few exceptions. EDWARDS and KUFFLER (1959) found very weak activity in the purely acidic γ-hydroxybutyric acid and in the purely basic agmatine, and we found 3:4-dihydroxyphenylacetic acid and tryptamine to be slightly inhibitory towards the one unusually sensitive crayfish preparation. ELLIOTT and FLOREY (1956) found fairly strong inhibitory activity in bromthymol blue, thymol and camphor; the block was a slowly developing one and may have been the result of a completely different process. Despite these exceptions, it would appear as if the presence of both an acidic and a

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basic group is essential for activity approximating that observed for GABA. The data are consistent with the concept of a 'two point' receptor site similar to that suggested by CURTIS and WATKINS (1960*b*) for mammalian spinal cord neurons; in their picture the negative and positive groups of an inhibitory compound form the two arms of a pincer approaching the cell receptor. A number of combinations of negative and positive groups are possible as evidenced in Tables 2, 3, and 4 so that the receptor sites do not appear to be peculiarly specific. From geometrical considerations of inhibitory materials, it appears that the two points on the receptor site must be about 4 Å apart. Branched or substituted derivatives of GABA and its homologues are presumably less active than the straight chain amino acids because the branching or substitution interferes with the two-pronged attack.

Although 3-hydroxytyramine (and the other active catecholamines) can assume an orientation somewhat similar to that presumed necessary for GABA-like activity, its inhibitory potential is of a much greater order of magnitude. Furthermore, 3-hydroxytyramine has a completely different response from GABA to the blocking agents, chlorpromazine, dibenzylene, picrotoxin, and RP-7843. The evidence therefore appears to favour a different type of receptor for the catecholamines. Imidazoleacetic acid is affected to a considerable extent by all the blocking agents and β-guanidinopropionic acid by all but RP-7843. Whether these compounds act on the same receptors as both GABA and 3-hydroxytyramine or whether still other receptors are involved cannot be determined from the evidence developed to date. It will be important to learn whether 3-hydroxytyramine acts on the dendrites, cell body, or axon. KUFFLER and EDWARDS (1957) have shown that GABA brings about changes in membrane conductance similar to those caused by stimulation of the inhibitory nerve supply to the stretch receptor neuron. There is no evidence, however, as to where this action of GABA occurs, and it is quite possible that other materials, acting on different receptor sites, would bring about the same effect.

As far as the catecholamines are concerned, this is, to our knowledge, the first report of a physiological preparation in which 3-hydroxytyramine is more active than either noradrenaline or adrenaline.

The significance of the extraordinary activity of 3-hydroxytyramine on the stretch receptor neuron remains to be assessed by much further work with other physiological systems. Interest in the physiological properties of 3-hydroxytyramine has lagged behind that of the other catecholamines. Its pharmacological activity on many smooth muscle preparations is weaker than that of adrenaline and noradrenaline and, furthermore, it has a well defined role as a precursor of these two substances. However, there is a disparity between the concentration of this compound and those of the catecholamines derived from it in various tissues; this disparity has led to the repeated suggestion that 3-hydroxytyramine may be an effector substance in its own right. Although 3-hydroxytyramine makes up only 2 per cent of the total catecholamine content of the adrenal medulla, it comprises 50 per cent of the total in nervous tissue (HOLTZ, 1959). Extracts of sympathetic nerves or of ganglia contain little or no adrenaline (SCHUMANN, 1958), so that the catecholamine content of these tissues appears to consist only of 3-hydroxytyramine and noradrenaline. A similar situation holds for brain, where little if any adrenaline can be found (HOLTZ, 1959). In brain, CARLSSON (1959) has found an important difference in the distribution of 3-hydroxytyramine and noradrenaline, with the former highest in the basal ganglia and the latter

in the hypothalamus. Furthermore, Carlsson has shown that reserpine depletes the brain of 3-hydroxytyramine as well as of noradrenaline, and that administration of L-dihydroxyphenylalanine is associated with a sharper increase in 3-hydroxytyramine than in noradrenaline. He has suggested that these findings may be correlated with the clinical similarity between basal ganglial disorders such as Parkinsonism and the extrapyramidal effects produced by administration of reserpine. Chlorpromazine, which is thought to block catecholamines rather than to deplete their levels, occasionally produces similar extrapyramidal side effects, and with RP-7843 extrapyramidal side effects are common.

Perhaps not coincidentally, areas of the brain high in 3-hydroxytyramine content have also been found to yield Factor I extracts of high activity (Florey and Florey, 1958). The crude Factor I preparation studied here does not mimic the actions of either GABA or 3-hydroxytyramine particularly closely, but acts as might a combination of the two. There was chromatographic evidence that this preparation of Factor I did contain GABA, but it seems extremely unlikely on both quantitative and qualitative grounds that the GABA was accounting for the majority of the activity. This is in contrast to the report of Bazemore, Elliott and Florey (1957) who concluded that most, if not all, of the activity of crude Factor I prepared in a similar manner could be accounted for by GABA. McLennan, however, has stressed that GABA, while behaving qualitatively like crude Factor I preparations on the stretch receptor neuron of the crayfish, does not duplicate their action in a number of other physiological situations (McLennan, 1957*b*, Honour and McLennan, 1960). Our efforts to isolate inhibitory materials other than GABA from crude Factor I preparations by solvent fractionation and preparative chromatography have led to an unexpected result. GABA, which is reasonably stable, appears to be generated spontaneously, presumably from glutamic acid, during the work-up of these preparations. Activity from some other, unidentified source, on the other hand, is unstable and disappears during chemical manipulation. On the basis of present evidence, it seems probable that a number of materials are contributing to the overall activity observed in crude Factor I preparations. Indeed, a number of substances now known to have inhibitory activity against the crayfish neuron are also known to be present in mammalian brain. If one of the substances contributing to the activity of crude Factor I preparations possessed an activity comparable to that of 3-hydroxytyramine, the amount contained would be so small as to render chromatographic identification extremely difficult.

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SUMMARY

The inhibitory activity of a number of simple and substituted aliphatic amino acids, catecholamines, indoles, imidazoles, and other nitrogen heterocycles has been tested on the stretch receptor neuron of the crayfish, *Pacifastacus leniusculus* (Dana).

All of the compounds which were found to have appreciable inhibitory activity had an acidic and basic function and could be oriented in such a way that these groups were approximately 4 Å apart and were sterically unhindered.

3-Hydroxytyramine was by far the most active of all the compounds tested, being about one hundred times as active as GABA. DL-Noradrenaline hydrochloride, imidazoleacetic acid, and β-guanidinopropionic acid all had activities comparable to that of GABA

Picrotoxin, dibenzylene, and chlorpromazine were assessed for their blocking action against the inhibitory effects of 3-hydroxytyramine, imidazoleacetic acid, β-guanidinopropionic acid, GABA, and a crude preparation of Factor I. Most of the activity of Factor I, imidazoleacetic acid, and β-guanidinopropionic acid was blocked by all three drugs; picrotoxin was only weakly effective against 3-hydroxytyramine but blocked about 60 per cent of the activity of GABA; and chlorpromazine and dibenzylene were ineffective against GABA but blocked 3-hydroxytyramine almost completely. These results suggest that more than one receptor site of the stretch receptor neuron is involved in the inhibitory effects observed. They also indicate that substances other than GABA were contributing to the inhibitory activity of this Factor I preparation.

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THE EFFECT OF PHYSICAL ACTIVITY ON THE INCORPORATION OF RADIOACTIVITY INTO PROTEINS OF THE CENTRAL NERVOUS SYSTEM, LIVER AND KIDNEY OF THE RAT, AFTER THE INTRACRANIAL INJECTION OF [^{35}S]METHIONINE*

JERZY KRAWCZYŃSKI
Central Clinical Laboratory of the 1st Clinical Hospital, Lublin, Poland

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IT HAS been shown (CZETWERNIKOW, 1955; GAITONDE and RICHTER, 1956; GOLUBCOWA and NAGRODOWA, 1955; GROMOWA, 1954; JAKOWLEW, 1956; ROSENGART and MASLOWA, 1957; WLADIMIROWA, 1956) that stimulation of the central nervous system (CNS) by different methods (electric shock, cardiazol insulin hypoglycaemia, hypoxia, auditory stimulation, conditioned reflex stimulation, forced swimming) is associated with a decrease of ATP in the brain and with a decreased turnover of phosphate in the RNA (SHAPOT, 1957) phosphoproteins (LISOWSKAJA and LIWANOWA, 1959) and phospholipids (ANSELL and DOHMEN, 1957) of the brain. It was also reported that under similar conditions the rate of incorporation of [^{35}S]methionine into the proteins of the CNS is decreased (SHAPOT, 1957).

Some investigators (GAITONDE and RICHTER, 1956; LISOWSKAJA, 1954, 1956; SHAPOT, 1957) have suggested that these different processes may be connected and that the decreased synthesis of certain compounds in the brain during stimulation of the CNS (a general inhibition of 'structural metabolism') may be due to a failure of the energy-supplying mechanisms owing to the increased demand for energy-rich compounds during physical activity (increased 'functional metabolism').

The investigations of VRBA (1955*a*, *b*, 1956*a*, *b*) suggest that stimulation of the CNS is associated with changes in the structure of proteins in the nervous tissue (hydrolysis of acid amide groups with the liberation of free ammonia). They thereby become more labile so that the catabolism of protein proceeds. The increased breakdown of the protein in the CNS must be compensated for by an increased synthesis either during the resting phase, or during the stimulation. The effect is an adaptive increase in the 'structural metabolism'. The existence of an 'adaptive phase' in animals active for several hours is confirmed by the experiments of LESZKIEWICZ (1959). He found that the extent of incorporation of ^{32}P into the phospholipids of the brain, liver and skeletal muscles depended on the length of time the animals were made to swim The incorporation was large initially, but after 10 hr it was similar to that of the control animals. The same author also observed that the modification of the phospholipid metabolism caused by prolonged swimming was less pronounced in animals which had been trained previously. Similar observations on the activity of proteolytic enzymes in skeletal muscle were made by ROGOZKIN (1959). CZAGOWIEC (1959)

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showed that hypercompensation may occur in the synthesis of certain compounds, e.g. glycogen, during the resting phase. It would appear that the alterations in the extent of the incorporation of labelled amino acids into the proteins of the stimulated CNS may depend on the duration of stimulation and on the biological characteristics of the different parts of the CNS. This may explain why the results of several investigators differ from the biochemical pattern which is generally accepted as characteristic of the stimulated CNS.

The experiments described here were designed to study the effect of physical activity (swimming for periods up to 4 hr) on the incorporation of amino acids in the proteins of the hemispheres, cerebellum, brain stem, liver and kidney of the rat after the intracranial injection of [^{35}S]methionine. The ATP content in the brain tissue of rats made to swim for 3 hr was also estimated.

METHODS

The experiments were carried out on white rats weighing 280–320 g in groups containing 3–4 animals. Each experiment was repeated 4–5 times and the mean values were estimated. [^{35}S]Methionine (4000 counts/min/g body weight) was administered by intracranial injection under light ether anaesthesia. The final volume of the fluid was either 0·1 or 0·2 ml. Thirty minutes after the injection of the isotope, the animals were placed in a container filled with warm water (33°–34°) and made to swim for 1, 2, 3 or 4 hr. At the end of the swimming period the animals were killed by decapitation, the organs were rapidly removed, and the protein was isolated by the methods previously described (Krawczyński *et al.*, 1960). The animals in the control groups were kept for 1, 2, 3 or 4 hr in an incubator at 33°–34° and then treated in a similar manner as the animals of the experimental groups. In all experiments, zero time was taken as 30 min after the injection of the isotope. The results were expressed as relative specific activities (RSA) of the proteins calculated as follows:

$$\text{RSA} = \frac{\text{counts/min/mg protein}}{\text{counts/min/ml soluble fraction}} \times 100$$

The soluble fraction was prepared as previously described (Krawczyński *et al.*, 1961). The ATP in the acid-soluble fraction was estimated according to the method described by Mieszkowa and Siewierin (1950). It was precipitated as the Hg-salt to isolate it from interfering hexosephosphates. All the operations in the estimation of the ATP content were conducted at low temperature.

RESULTS

Changes in relative specific activities

Three hours after the injection of [^{35}S] methionine the relative specific activities of the proteins reached their maximal values in both the control and experimental groups (Table 1).

The pattern of change in RSA varied in each of the parts investigated.

Brain hemispheres. One hour after the injection of [^{35}S]methionine, the RSA of the proteins was 57 per cent higher in the experimental animals than in the controls. This difference decreased with time and after swimming for 3 hr the RSA was lower in the experimental rats. After 4 hr there was little difference in the two groups.

Cerebellum. After 1 hr the RSA of the proteins in the swimming rats was lower than in resting animals, and after 2 hr there was little difference. After 3 hr the RSA was again lower in the swimming animals but at 4 hr was higher than that of the control.

Brain-stem. The RSA of the proteins was higher at all times in the experimental animals. The differences were greater after swimming for 2 and 4 hr and least marked after 3 hr.

TABLE 1.—CHANGES IN THE RELATIVE SPECIFIC ACTIVITIES OF THE PROTEINS OF THE CENTRAL NERVOUS SYSTEM, KIDNEY AND LIVER OF RATS DURING PHYSICAL ACTIVITY (SWIMMING)

	Period of activity or rest (hours)											
	1			2			3			4		
	RSA Mean ± S.D.		Change in RSA as per cent of control	RSA Mean ± S.D.		Change in RSA as per cent of control	RSA Mean ± S.D.		Change in RSA as per cent of control	RSA Mean ± S.D.		Change in RSA as per cent of control
	Resting animals	Swimming animals		Resting animals	Swimming animals		Resting animals	Swimming animals		Resting animals	Swimming animals	
Brain hemispheres	4·84 ± 1·26	7·62 ± 1·75	+57·4* $P < 0·01$	14·96 ± 3·85	17·07 ± 4·24	+13·7* $P < 0·05$	27·15 ± 6·09	23·37 ± 5·33	−14·0* $P < 0·05$	20·48 ± 5·55	20·46 ± 3·42	−0·1 $P > 0·1$
Cerebellum	10·79 ± 2·70	9·62 ± 2·47	−10·8* $P < 0·05$	21·04 ± 4·66	21·10 ± 4·88	+0·3 $P > 0·1$	27·81 ± 5·41	25·88 ± 5·59	−6·9* $P < 0·05$	19·50 ± 4·91	21·30 ± 4·62	+9·2* $P < 0·05$
Brain-stem	7·13 ± 1·35	7·89 ± 1·43	+10·6* $P < 0·05$	9·81 ± 2·27	11·72 ± 2·83	+19·4* $P < 0·02$	17·00 ± 4·63	17·81 ± 4·60	+4·8 $P < 0·1$	10·21 ± 1·47	12·35 ± 1·97	+20·9* $P < 0·02$
Kidney	12·81 ± 3·21	14·23 ± 3·75	+11·1* $P < 0·05$	15·66 ± 3·08	15·58 ± 3·60	−0·6 $P > 0·1$	25·64 ± 5·82	27·87 ± 5·57	+8·7* $P < 0·05$	20·18 ± 3·70	23·27 ± 3·93	+15·3* $P < 0·05$
Liver	27·68 ± 5·32	20·22 ± 4·41	−26·9* $P < 0·02$	39·10 ± 8·30	25·52 ± 4·14	−34·8* $P < 0·01$	59·14 ± 11·01	43·16 ± 9·02	−27·1* $P < 0·01$	57·78 ± 9·36	32·83 ± 6·00	−43·2* $P < 0·01$
No. of animals	15	13		20	18		20	16		20	15	

Zero time is taken as 30 min after the intracranial injection of [^{35}S]methionine (4000 counts per min per g.)
* Statistically significant at the level 0·05.

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Kidney. The RSA of the proteins was higher after swimming for 1, 3 and 4 hr and only slightly lower after 2 hr.

Liver. The RSA of the liver proteins was significantly lower at all times in the rats made to swim. The differences were particularly marked after 2 and 4 hr.

Changes in ATP and relative specific activity of whole brain

After 3 hr the RSA of proteins in the whole brain was lower in the swimming animals than in the controls and the ATP content of the acid-soluble fraction of whole brain was also lower (Table 2).

TABLE 2.—CHANGES IN THE RELATIVE SPECIFIC ACTIVITIES OF WHOLE BRAIN PROTEINS AND IN THE ATP CONTENT OF WHOLE BRAIN AFTER 3 HR OF SWIMMING

[^{35}S]Methionine (4000 counts/min per g) was injected intracranially into rats weighing about 300 g. ATP content expressed as mg P_7/min per g of wet tissue. Mean values ± S.D.

	RSA			ATP		
	Group		Changes in RSA as per cent of control	Group		Changes in ATP content as per cent of control
	Resting	Swimming		Resting	Swimming	
Whole brain	23·97 ± 4·37	22·35 ± 5·14	−6·38 $P < 0.05$	0·117 ± 0·030	0·088 ± 0·018	−23·5 $P < 0.02$
No. of animals	25	20		25	20	

DISCUSSION

The data on the changes in the relative specific activities of the brain proteins with time after the intracranial injection of [^{35}S]methionine agree well with the results recently obtained by CLOUET and RICHTER (1959). The RSA of proteins in the control animals were independent of the dose of labelled amino acid administered, which varied between 250 and 5000 counts/min per g.

The changes observed in the RSA of the proteins cannot be fully explained on the basis of views previously expressed in this paper. Some of the values might be interpreted as due to competition between the structural and functional metabolism in the tissues. An inhibition of the structural metabolism is suggested by a decrease in the RSA of the tissue proteins. The inhibition might arise from an increased utilization of energy stores in the cells as a result of increased physical activity. It is relevant that the decrease in the RSA of the proteins of the CNS after 3 hr of swimming was associated with a decrease in the amount of ATP in brain tissue. The increase in the RSA of the proteins from certain organs, which depends on the duration of the stimulation, may indicate an increase in the 'structural metabolism'.

As judged by the pronounced decrease in the RSA of the proteins in the liver of rats that were swimming, the protein metabolism was altered to a much greater extent in the liver than in other organs . These facts are fully comprehensible when the central role of the liver in all the metabolic processes in the animal organism is taken into account.

We must take into consideration the role of the adrenal cortex in the mechanism of the observed phenomena. Because the swimming animals can be considered as being under stress, it is right to regard the observed changes in protein metabolism in the CNS, liver and kidney as evidence of the different phases of the 'general adaptation syndrome' (SELYE, 1947).

SUMMARY

The effect of swimming for periods of 1, 2, 3 and 4 hr on the RSA of the proteins isolated from brain hemispheres, cerebellum, brain-stem, liver and kidney was investigated in rats after intracranial injection of [^{35}S]methionine. The pattern of change with time varied in each of the tissues examined.

The possible reasons for the changes in the RSA of the proteins are discussed.

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THE UPTAKE OF CIRCULATING [^{3}H]NOREPINEPHRINE BY THE PITUITARY GLAND AND VARIOUS AREAS OF THE BRAIN

H. WEIL-MALHERBE, L. G. WHITBY and JULIUS AXELROD
National Institute of Mental Health, Bethesda, Maryland, U.S.A.

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IT WAS shown previously (WEIL-MALHERBE, AXELROD and TOMCHICK, 1959) that circulating [^{3}H]epinephrine did not cross the blood-brain barrier to any significant extent except in the hypothalamic region. Even there the uptake was slight in comparison with extracerebral tissues (AXELROD, WEIL-MALHERBE and TOMCHICK, 1959).

These experiments have now been extended to [^{3}H]norepinephrine; furthermore the effect of a series of drugs on the uptake of circulating [^{3}H]norepinephrine by the pituitary gland and by various areas of the brain has been studied. Preliminary accounts of this work have been presented at two symposia (WEIL-MALHERBE, 1960; WEIL-MALHERBE, WHITBY and AXELROD, 1961).

METHODS

Detailed accounts of the methods and materials used were given in previous publications (WEIL-MALHERBE *et al.*, 1959; AXELROD *et al.*, 1959; WHITBY, AXELROD and WEIL-MALHERBE, 1961). Briefly, DL-β[^{3}H]norepinephrine, (3·44 mC/mg), in doses of 25 μg of free base per kg, was administered to cats through the femoral vein either by rapid injection or by slow infusion over a period of 30 min. The cats were decapitated at different times after the injection and brain and pituitary gland were removed. For the preparation of the hypothalamus an area of roughly rhombic shape, bordered caudally by the pedunculi and extending rostrally slightly beyond the optic chiasma, was excised from the base of the brain to a depth of 2–3 mm. It included the mammillary bodies and usually weighed 150–200 mg. The weighed tissue samples were homogenized in 5–10 vol of 0·1 N-HCl and, after the addition of 0·1 vol of 2% ascorbic acid and 0·1 vol of 1% sodium ethylenediaminetetraacetate, were adjusted to pH 8·4. The mixture was centrifuged at about 10,000 **g** and 0° in a Servall centrifuge with a SS-1 head, and the supernatant solution was purified by chromatography on columns of alumina. The eluate containing [^{3}H]norepinephrine was evaporated to dryness, the residue was dissolved in a small amount of methanol and ethanol, and the radioactivity of the sample was determined in a Packard liquid scintillation spectrophotometer.

Blood was collected at decapitation in a heparinized container. Plasma was diluted with an equal volume of 0·2 M-sodium acetate and adjusted to pH 8·4 with 0·5 N-sodium carbonate before being passed through a column of alumina. The eluate was treated then in the same way as that from the tissue homogenates.

Evidence for the specificity of the procedure was presented by WHITBY *et al.* (1961).

Corrections for counting efficiency were carried out by adding internal standards in most of the later experiments. The corrections did not greatly affect the ratio of tissue to plasma concentrations. Where the correction affected the result, the ratio was decreased; the difference between corrected and uncorrected results rarely exceeded 30 per cent.

RESULTS

Uptake of [^{3}H]*norepinephrine by the pituitary gland and by various areas of the brain*

The concentration of [^{3}H]norepinephrine in the pituitary gland and in different areas of the brain at 2, 60 and 120 min after a rapid injection and at the end of a 30-min infusion is shown in Table 1, in absolute terms (μg/kg wet weight) and relative

to the concentration in plasma. Two minutes after a rapid injection the pituitary gland contained radioactive norepinephrine in high concentration, exceeding that in plasma. One experiment in which the anterior and posterior pituitary were analysed separately showed that uptake was greater in the anterior portion of the gland. In the course of the next two hours, the concentration of [^{3}H]norepinephrine declined slowly in the pituitary gland while the concentration relative to that of plasma increased rapidly. In this respect, the pituitary gland resembles other extracerebral tissues, such as heart and spleen, which also take up the hormone rapidly and retain it more firmly than plasma (WHITBY *et al.*, 1961). At the end of a 30 min infusion, the uptake in the anterior pituitary also exceeded that in the posterior pituitary, but the difference tended to disappear after 2 hr.

In most areas of the brain, with the exception of the hypothalamus, the highest concentration of [^{3}H]norepinephrine was found 2 min after a rapid injection. The concentration subsequently declined at a rate similar to that observed in plasma. The ratio of tissue/plasma concentrations therefore remained approximately constant and, on the whole, did not appreciably exceed the value to be expected on the basis of the blood content of brain tissue. This, according to OEFF and KOENIG (1955), amounts to 2·4 per cent for the brain of a rat which has not been exsanguinated. Somewhat lower values were reported by DAVSON and SPAZIANI (1959) and by NAIR, PALM and ROTH (1960). The latter authors determined the vascularity of different areas of rat brain and found a blood content of 0·7–1·0 per cent for predominantly white matter and 1·7–2·2 per cent for predominantly grey matter.

Two hours after a rapid injection, the ratio of radioactivity in the medulla to that in plasma was significantly greater than the critical 2 per cent level; this result probably indicates a slow uptake. This is not surprising since the tissue samples included the area postrema which is assumed to be outside the blood-brain barrier. In other brain areas, with the exception of the hypothalamus, the radioactivity did not significantly exceed 2 per cent of the plasma activity.

The hypothalamus differed from other brain areas in that it contained radioactive material in concentrations substantially higher than that corresponding to 2 per cent of the plasma activity in all but the experiments terminated 2 min after a rapid injection. In absolute figures, the uptake was highest at the end of a 30 min infusion; it was significantly higher at 1 hr than at 2 min after a rapid injection and declined only slightly during the following hour. The uptake of [^{3}H]norepinephrine by the hypothalamus was thus considerably slower than in extracerebral tissues where the highest uptake was generally found within 2 min of the injection (WHITBY *et al.*, 1961). The concentration in the hypothalamus after 1 hr was 31 per cent of that in the plasma. The excess over the 2 per cent level is highly significant ($P < 0{\cdot}001$). It was even higher after 2 hr (66 per cent of the plasma concentration) but wide variations in the individual results gave a high standard error. By excluding two extreme results, amounting to 174 and 207 per cent of the plasma concentration, a mean percentage of $16{\cdot}7 \pm 5{\cdot}60$ of the plasma concentration was obtained, a result which is significantly different from 2 per cent at the 0·05 level of probability.

Drug effects

The effect of a number of drugs on the disposition of [^{3}H]norepinephrine in extracerebral tissues was studied by AXELROD, WHITBY and HERTTING (1961) and HERTTING,

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TABLE 1.— CONCENTRATION OF [^{3}H]NOREPINEPHRINE IN CAT PITUITARY AND VARIOUS AREAS OF CAT BRAIN AFTER INTRAVENOUS ADMINISTRATION

	Time after injection							
	2 min		30 min		1 hr		2 hr	
	μg/kg	% of plasma concentration	μg/kg	% of plasma concentration	μg/kg	% of plasma concentration	μg/kg	% of plasma concentration
Pituitary (anterior + posterior)	36·3 ± 4·72 (3)	159·3 ± 33·6 (3)			26·6 ± 1·59 (18)	560 ± 18·0 (18)	20·8 ± 5·42 (3)	1540 ± 398 (3)
Anterior pituitary	67·7 (1)	297 (1)	38·9 (2)	178 (2)			27·0 ± 12·7 (5)	818 ± 226 (5)
Posterior pituitary	25·1 (1)	110 (1)	19·2 (2)	87·2 (2)			22·4 ± 2·98 (5)	905 ± 203 (5)
Cerebellum	0·45 ± 0·16 (4)	1·77 ± 0·44 (4)	0·175 (2)	0·80 (2)			0·080 ± 0·016 (8)	4·7 ± 1·95 (8)
Hypothalamus	0·59 ± 0·17 (4)	2·36 ± 0·53 (4)	1·66 (2)	8·2 (2)	1·49 ± 0·34 (18)	30·8 ± 6·8 (18)	1·23 ± 0·63 (7)	66·4 ± 32·5 (7)
Cerebral cortex	0·37 ± 0·12 (4)	1·46 ± 0·36 (4)	0·26 (2)	1·24 (2)	0·087 ± 0·0090 (18)	1·68 ± 0·15 (18)	0·060 ± 0·0081 (8)	3·0 ± 0·57 (8)
Medulla	0·52 ± 0·17 (4)	2·02 ± 0·51 (4)	0·18 (2)	0·84 (2)	0·109 ± 0·0094 (18)	2·24 ± 0·27 (18)	0·095 ± 0·013 (8)	5·1 ± 1·16 (8)
Pons	0·33 ± 0·103 (4)	1·28 ± 0·29 (4)	0·18 (2)	0·82 (2)			0·057 ± 0·010 (8)	3·6 ± 1·23 (8)
Mesencephalon	0·38 ± 0·11 (4)	1·50 ± 0·30 (4)	0·20 (2)	0·92 (2)			0·061 ± 0·0085 (7)	3·0 ± 0·88 (7)
Basal ganglia	0·37 ± 0·06 (4)	1·62 ± 0·09 (4)	0·15 (2)	0·72 (2)			0·077 ± 0·0205 (8)	4·5 ± 1·74 (8)
Plasma (μg/l.)	23·9 ± 2·76 (4)		21·5 (2)		6·68 ± 1·13 (18)		2·25 ± 0·48 (8)	

The figures are means ± S.E.M. (No. of expts).
* Infusion experiments (see text).

AXELROD and WHITBY (1961). The effect of these drugs on the uptake of [^{3}H]norepinephrine by selected brain areas and by the pituitary gland will be reported in this paper.

In the drug experiments, [^{3}H]norepinephrine was administered by rapid intravenous injection; the cats were decapitated 1 hr later. The brain areas studied were the hypothalamus, the medulla, and the cerebral cortex.

Details of the treatment schedules are shown in Table 2. Briefly, long-acting drugs were injected hours or days before the injection of [^{3}H]norepinephrine, usually in repeated doses. Short-acting drugs were given shortly before the injection of norepinephrine.

TABLE 2.—DOSAGES AND ADMINISTRATION SCHEDULE OF DRUGS

Drug	Amount (mg/kg)	Route	Time before (−) or after (+) the injection of [^{3}H]norepinephrine
Reserpine	3	i.p.	(1) −24 hr (2) −1 hr
Chlorpromazine (1)	20	i.p.	−24 hr
(2)	5	i.v.	−1 hr
Imipramine	20	i.p.	(1) −24 hr (2) −5 min
Pyrogallol	50	i.v.	(1) −3 min (2) +30 min
Cocaine	5	i.v.	−10 min
Iproniazid	50	i.p.	(1) −48 hr (2) −24 hr (3) −12 hr
Amphetamine	10	i.v.	−10 min
β-Phenyl*iso*propyl-hydrazine (Catron)	1·5	subcutaneous	(1) −72 hr (2) −48 hr (3) −24 hr
Dibenzylene	20	i.v.	−90 min
Dichloro*iso*proterenol (DCI)	10	i.v.	−15 min
Tyramine	10	i.v. infusion over 5 min	−10 min
Ouabaine	0·05	i.v. infusion over 5 min	−10 min
Guanethidine	15	i.v.	−30 min
Hexamethonium	10	i.v.	−10 min
Regitine	5	i.v.	−10 min
TM 10 B	10	i.v.	−20 min

The results of the drug experiments are shown in Table 3. Reserpine blocked the uptake of norepinephrine in the pituitary gland, in accordance with its effect in all other extracerebral tissues examined (AXELROD *et al.*, 1961). However, reserpine had surprisingly little effect on the uptake in the hypothalamus, in marked contrast to its well-known releasing effect on endogenous brain catecholamines. Chlorpromazine which depressed the uptake of norepinephrine in most extracerebral tissues (AXELROD *et al.*, 1961) did not change the uptake in the pituitary gland although it significantly inhibited the uptake in the hypothalamus, both in absolute terms and relative to plasma. Imipramine, cocaine, amphetamine, tyramine and dibenzylene resembled chlorpromazine in their effect on extracerebral tissues, yet with the exception of dibenzylene, they did not significantly retard uptake in the pituitary. Of these drugs, only amphetamine depressed the uptake in the hypothalamus. Tyramine and hexamethonium slightly increased the absolute concentration of [^{3}H]norepinephrine in

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TABLE 3.—EFFECT OF DRUGS ON THE UPTAKE OF [^{3}H]NOREPINEPHRINE

Drug	No. of Expts	Pituitary gland		Hypothalamus		Medulla		Cortex	
		μg/kg	% of plasma	μg/kg	% of plasma	μg/kg	% of plasma	μg/kg	% of plasma
0	18	26·6 ± 1·59	560 ± 18·0	1·49 ± 0·34	30·8 ± 6·8	0·109 ± 0·0094	2·24 ± 0·27	0·087 ± 0·0090	1·68 ± 0·15
Reserpine	3	8·1 ± 1·68‡	128 ± 33·4‡	1·17 ± 0·41	18·7 ± 6·9	0·166 ± 0·086	2·54 ± 0·17	0·142 ± 0·033	2·13 ± 0·34
Chlorpromazine	4	34·0 ± 4·73	870 ± 175	0·51 ± 0·06†	12·8 ± 1·6*	0·125 ± 0·048	2·55 ± 0·77	0·184 ± 0·10	4·77 ± 2·70
Imipramine	4	25·4 ± 4·13	748 ± 284	2·38 ± 1·88	43·3 ± 30·2	0·122 ± 0·028	2·87 ± 0·16	0·243 ± 0·11	6·32 ± 2·97
Pyrogallol	3	45·2 ± 5·38†	381 ± 39·2‡	2·14 ± 0·83	18·5 ± 7·8	0·463 ± 0·21	4·02 ± 1·87	0·97 ± 0·28†	8·4 ± 2·55*
Cocaine	4	24·4 ± 1·43	500 ± 29·5	0·76 ± 0·17	27·1 ± 6·1	0·100 ± 0·023	3·27 ± 0·51	0·058 ± 0·008*	2·3 ± 0·20*
Iproniazid	3	41·7 ± 18·2	965 ± 411	0·66 ± 0·24	15·3 ± 5·4	0·080 ± 0·038	1·86 ± 0·87	0·076 ± 0·047	1·77 ± 1·07
Amphetamine	3	17·5 ± 5·1	595 ± 235	0·39 ± 0·11†	13·4 ± 5·5	0·080 ± 0·013	2·67 ± 0·72	0·062 ± 0·015	2·0 ± 0·54
β-Phenyl*iso*-propylhydrazine (Catron)	3	43·3 ± 2·77‡	784 ± 179	1·21 ± 0·72	25·4 ± 18·0	0·14 ± 0·038	2·68 ± 1·16	0·104 ± 0·026	1·99 ± 0·81
Dibenzylene	3	16·4 ± 2·73†	285 ± 103*	0·94 ± 0·45	16·3 ± 7·8	0·11 ± 0·019	1·77 ± 0·36	0·081 ± 0·020	1·25 ± 0·26
Dichloro*iso*-proterenol (DCI)	2	16·8(16·3, 17·4)	510(566; 454)	0·99(0·89; 1·09)	29·6(30·9; 28·4)	0·17(0·039; 0·298)	4·57(1·34, 7·8)	0·23(0·39, 0·071)	7·78(13·7, 1·86)
Tyramine	3	36·7 ± 9·7	380 ± 102	3·7 ± 2·2	45·1 ± 30·4	0·188 ± 0·025†	1·84 ± 0·23	0·59 ± 0·48	1·28 ± 0·22
Ouabaine	3	34·0 ± 9·8	463 ± 53	1·5 ± 0·53	18·2 ± 4·2	0·119 ± 0·069	1·44 ± 0·55	0·097 ± 0·024	1·31 ± 0·22
Guanethidine	3	40·5 ± 15·1	740 ± 133	1·29 ± 0·06	22·5 ± 5·5	0·18 ± 0·017	3·2 ± 0·77	0·14 ± 0·029	2·29 ± 0·15*
Hexamethonium	3	37·3 ± 8·8	452 ± 144	2·66 ± 0·60	27·6 ± 11·2	0·162 ± 0·0067†	1·9 ± 0·16	0·23 ± 0·077	2·5 ± 0·53
Regitine	4	38·3 ± 6·8	326 ± 126	1·97 ± 0·59	16·2 ± 6·4	0·29 ± 0·073	2·08 ± 0·41	0·10 ± 0·015	0·82 ± 0·21†
TM 10	3	41·1 ± 7·8	297 ± 55‡	0·73 ± 0·02	5·4 ± 0·68†	0·08 ± 0·03	0·60 ± 0·26‡	0·16 ± 0·052	1·17 ± 0·38

The figures are means ± S.E.M.
Differences from control * $P < 0·05 > 0·01$
† $P < 0·01 > 0·001$
‡ $P < 0·001$.

the medulla without affecting the concentration relative to plasma; the increase can therefore be attributed to the higher plasma level. Cocaine, while increasing the concentration relative to plasma, slightly but significantly reduced the absolute concentration in the cerebral cortex.

Pyrogallol which increased the [^{3}H]norepinephrine uptake in extracerebral tissues (HERTTING *et al.*, 1961) had a similar effect in the pituitary gland. Iproniazid also increased the uptake of [^{3}H]norepinephrine in the pituitary gland but there was a large scatter in the results and the effect did not reach the level of significance. Catron, another inhibitor of monoamine oxidase, caused a similar increase which was significant. Among extracerebral tissues, only skeletal muscle showed an increased uptake of [^{3}H]norepinephrine after the administration of Catron. In none of these experiments was the concentration of [^{3}H]norepinephrine significantly increased relative to plasma; in the case of pyrogallol, it was actually decreased. The increased uptake was therefore presumably the result of a higher and more sustained level of [^{3}H]norepinephrine in the plasma.

Pyrogallol, iproniazid and Catron were without effect on the uptake of [^{3}H]norepinephrine in the hypothalamus. Pyrogallol, however, significantly increased the uptake in cerebral cortex, both as regards the absolute concentration and the concentration relative to plasma. Since pyrogallol is an inhibitor of catechol-O-methyl transferase (AXELROD and LAROCHE, 1959), the possibility had to be considered that the unmethylated metabolite, 3:4-dihydroxymandelic acid, was present in the blood in increased amounts and penetrated into the cerebral cortex. Experiments designed to detect the presence of [^{3}H]dihydroxymandelic acid in the plasma of animals treated with [^{3}H]norepinephrine and pyrogallol were, however, negative (HERTTING *et al.*, 1961).

Some other significant changes were observed with respect to the concentrations of [^{3}H]norepinephrine relative to plasma although the absolute concentrations in the tissues were not affected. Thus TM 10B caused decreases in the relative concentrations in pituitary gland, hypothalamus and medulla, and Regitine had a similar effect in cerebral cortex. This might indicate an inhibition of tissue uptake and a rise in plasma levels, presumably as a consequence of the diminished tissue uptake.

DISCUSSION

It is generally recognized that the blood-brain barrier does not include certain appendages of the brain, such as the choroid plexus, the posterior pituitary, the pineal gland and the area postrema. Histological evidence further suggests that the blood-brain barrier, even where it does function, is not uniformly effective in different areas of the brain; in particular, the central grey substance is known to be more accessible to vital stains than the rest of the brain (WELLS and CARMICHAEL, 1930; WISLOCKI and KING, 1936; WISLOCKI and LEDUC, 1952). Recently, BRODIE, TITUS and WILSON (1960) reported that greater quantities of certain drugs were absorbed from the blood by the intercolumnar tubercle than by the cortex or the hypothalamus.

The experiments described in this communication have shown that circulating [^{3}H]norepinephrine is capable of entering the hypothalamus at a faster rate than other areas of the brain; similar results were previously obtained with [^{3}H]epinephrine (WEIL-MALHERBE *et al.*, 1959). Even though the uptake of [^{3}H]norepinephrine in the hypothalamus was considerably greater than in other brain areas, it was slow

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compared with extracerebral tissues. While a time interval of 2 min was generally sufficient for extracerebral tissues to reach the peak of their [^{3}H]norepinephrine concentration (WHITBY *et al.*, 1961), the uptake in the hypothalamus at that interval was similar to that in the rest of the brain, most of it being accounted for by the blood content of the tissue. Maximal uptake in the hypothalamus was only observed after about 30 min.

Though it would have been desirable to determine the blood content of individual tissue samples, this was found to be impracticable by simple methods owing to the need for rapid acidification of the homogenate. The assumption of a blood content of 2 per cent is undoubtedly only a rough and arbitrary approximation; the true value is probably less than 2 per cent for such regions as the hypothalamus and the medulla which are rich in white matter. The possibility of a slow uptake of [^{3}H]norepinephrine by all areas of the brain therefore cannot be ruled out; indeed, it would be surprising if it were otherwise. What is important, however, is the fact that, compared with other brain areas, the hypothalamus is in a class by itself. This is of particular interest in view of the fact that the highest concentrations of sympathetic centres and of endogenous norepinephrine (VOGT, 1954) are found in this area.

The dose of [^{3}H]norepinephrine administered in these experiments was intentionally limited to simulate a discharge which might be expected to occur under physiological conditions. The concentrations of circulating [^{3}H]norepinephrine which falls rapidly during the first few minutes but slowly thereafter (WHITBY *et al.*, 1961) was, therefore, during the greater part of the experiment well below that of endogenous norepinephrine in most areas of the brain. Accordingly, the amount of labelled norepinephrine entering the hypothalamus, even at its highest concentration, was only a minute fraction, about 0·2 per cent, of the endogenous norepinephrine concentration in the hypothalamus. It is possible that entry took place through an exchange mechanism rather than by an active transport leading to a net increase. If such is the case, the smaller amount of isotope entering other brain areas may be related to the fact that, similarly, their endogenous norepinephrine concentrations are proportionately smaller than that in the hypothalamus. On the other hand, the concentration of endogenous norepinephrine which may exist largely in a bound or inactive form may bear little relation to the sensitivity of suitably placed receptors to circulating norepinephrine.

DRAŠKOCI, FELDBERG and HARANATH (1960) found that epinephrine, perfused intravenously at a rate of 40 μg/kg per min, appeared in the perfusate of cerebral ventricles and of the subarachnoidal space. This would suggest that epinephrine is capable of crossing the blood-cerebrospinal fluid barrier. In one experiment in which we managed to obtain cerebrospinal fluid uncontaminated by blood, the concentration of [^{3}H]epinephrine was only 0·5 per cent of that of plasma (WEIL-MALHERBE *et al.*, 1959). It is however difficult to compare the experiments of DRAŠKOCI *et al.* and ours since so much larger concentrations of catecholamines were used in the former (see FELDBERG, 1961).

The results obtained with hypothalamic extracts were more variable than those of extracts of other brain areas, especially in the experiments where the animal was killed 2 hr after the injection. The scatter may be due to experimental error; it is feasible, for instance, that the hypothalamic region contains only a small active area which might be contaminated by variable amounts of inactive tissue in different

experiments. It is equally possible, however, that the scatter corresponds to individual differences; in that case one would have to assume that entry of norepinephrine into the hypothalamus is governed by factors, possibly of hormonal nature, which were inadequately controlled in our experiments.

Since catecholamines do not easily cross the blood-brain barrier, the question arises how their 'central effects' are to be explained. Such effects may be indirect, mediated by the vasomotor action of catecholamines, or they may be the result of a direct interaction with nerve cells. Our observations suggest that, if such an interaction occurs, the receptors are most likely to be situated in the hypothalamus. It has indeed been shown that the area from which circulating epinephrine is capable of eliciting arousal responses is a narrow region limited to the anterior part of the mesencephalon and the posterior part of the hypothalamus (BONVALLET, DELL and HIEBEL, 1954). It still remains to be demonstrated that the rate of transfer of catecholamines from blood to hypothalamus is adequate. The problem of central adrenergic mechanisms has been fully discussed by DELL (1960).

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Many of the drug effects observed in extracerebral tissues (AXELROD *et al.*, 1961; HERTTING *et al.*, 1961) were absent in the pituitary gland. Thus, chlorpromazine, imipramine, cocaine, amphetamine, tyramine and guanethidine greatly reduced the uptake of [^{3}H]norepinephrine in the heart, yet they were without effect in the pituitary gland. This difference of response seems to indicate differences in the organization of binding sites in different tissues. The inhibitory effects of reserpine and dibenzylene and the stimulatory effects of pyrogallol and Catron are however the same in the pituitary as in other extracerebral tissues.

The passage of [^{3}H]norepinephrine into the brain is, on the whole, scarcely affected by drugs. Uptake in the hypothalamus was inhibited by chlorpromazine and amphetamine, in accordance with the action of these drugs on the heart. The similarity of their action on the uptake of norepinephrine is strange in view of the contrast of their other pharmacological effects. None of the other drugs significantly affected the uptake of norepinephrine in the hypothalamus. It is particularly surprising that reserpine which has a strong blocking action in all extracerebral tissues did not prevent the hypothalamic uptake. Reserpine has been claimed to increase the permeability of the blood-brain barrier towards phosphate (QUADBECK and SCHMITT, 1959) and towards norepinephrine (ROSENBLATT, CHANLEY, SOBOTKA and KAUFMAN, 1960). This effect may, to some extent, have counterbalanced the blocking action of reserpine in the hypothalamus; but other parts of the brain showed no sign of an increased influx of norepinephrine.

Pyrogallol was the only drug which apparently increased the permeability of the blood-brain barrier for norepinephrine. Though without effect in the hypothalamus, it increased the uptake of norepinephrine in the medulla and cerebral cortex, in the latter to a significant degree, The possibility cannot be ruled out that there was interference by a non-methylated metabolite which crossed the blood-brain barrier more readily than norepinephrine, yet no evidence for the presence of such a metabolite in plasma was detected. Moreover, if this was the mechanism of the pyrogallol effect, it should also have affected the hypothalamus but this was not the case. A more likely explanation is that pyrogallol caused a local hyperaemia; a considerable engorgement of the blood vessels on the surface of the cerebral hemispheres was indeed observed in animals treated with pyrogallol.

SUMMARY

DL-β[^{3}H]Norepinephrine, in doses of 25 μg/kg, was administered to cats, usually by rapid intravenous injection. The concentration of [^{3}H]norepinephrine was determined in the pituitary gland and in various areas of the brain, at different intervals after the injection.

The concentration of [^{3}H]norepinephrine in the pituitary rose to a level exceeding the concentration in plasma within 2 min and declined only slightly during the following 2 hr. Initially, more norepinephrine was taken up by the anterior than by the posterior pituitary gland.

The concentrations of [^{3}H]norepinephrine in various areas of the brain were generally within the limits to be expected from the blood content of the tissue. The only exception was the hypothalamus where these limits were appreciably exceeded. Compared with most extracerebral tissues, however, the rate of uptake was slow even in the hypothalamus. The uptake of labelled norepinephrine in the hypothalamus was only about 0·2 per cent of the endogenous norepinephrine content.

The effect of a series of drugs on the uptake of circulating norepinephrine in the pituitary gland and in various brain areas was studied. Reserpine blocked the uptake of norepinephrine by the pituitary, but had no effect on its passage into the hypothalamus. Chlorpromazine, imipramine, cocaine, amphetamine and tyramine, all of which greatly depressed the uptake of norepinephrine by the heart, did not significantly affect the uptake in the pituitary; of these drugs only chlorpromazine and amphetamine inhibited the uptake in the hypothalamus.

Increased concentrations of [^{3}H]norepinephrine were found in the pituitary after treatment with pyrogallol or with inhibitors of monoamine oxidase; they were probably secondary to increased plasma levels. Pyrogallol also caused an increase of the [^{3}H]norepinephrine concentration in cerebral cortex; it is suggested that this effect is due to local hyperaemia.

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THE RELEASE OF AMMONIA FROM RAT BRAIN PROTEINS DURING ACID HYDROLYSIS

R. VRBA and KUNJLATA KOTHARY
Medical Research Council, Neuropsychiatric Research Unit,
Woodmansterne Road, Carshalton, Surrey

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THE AMIDE groups of proteins are hydrolysed by acids under mild conditions before there is any extensive hydrolysis of the peptide bonds: OSBORNE and NOLAN (1920) found that 1% hydrochloric acid at 100° released 97 per cent of the amide ammonia from gliadin, while only 3·7 per cent of the peptide bonds were hydrolysed. The ammonia released under these conditions comes mainly from the amide side-chains of glutamine and asparagine; ammonia formation from serine and threonine by deamination is relatively slow and does not become established until the hydrolysis of the protein is far advanced.

Free glutamine liberates ammonia more readily than does asparagine, since it forms the cyclic product pyrrolidone carboxylic acid; but the amide groups of protein-bound glutamine and asparagine are apparently hydrolysed at similar rates (LEACH, 1953). The release of ammonia from ovalbumin proceeds smoothly as a first-order reaction; it has therefore been concluded that only one type of amide group is present (SHORE, WILSON and STUECK, 1935-6.) On the other hand GERSHENOVICH and KRICHEVSKAYA (1960) have recently reported that brain proteins contain amide groups of two types, (a) 'labile' and (b) 'firmly-bound,' which differ in physiological behaviour as well as in lability to hydrolysis by acids.

The present investigation was carried out to obtain further information about the amide groups in brain proteins and to see if the presence of amide groups differing in lability could be confirmed.

METHODS

Preparation of rat-brain proteins. Wistar albino rats were decapitated and the brains were quickly removed and frozen in liquid air. The pooled brains of 30 rats were homogenized in a homogenizer (ALDRIDGE, EMERY and STREET, 1960) in 200 ml 0·25 M-sucrose at 0°. For most experiments the suspension was precipitated with 50% trichloroacetic acid (TCA) at 0° (final concentration 10% TCA, w/v) and a brain protein fraction was prepared under mild conditions by repeated extraction of lipids at room temperature with acetone–ethanol ether (3:1, v/v) chloroform–methanol (2:1, v/v) and ether as described by VRBA, FOLBERGROVÁ and KANTŮREK (1957). The product was dried overnight in the air at 20°. The resulting dry protein fraction was similar to that used by GERSHENOVICH and KRICHEVSKAYA (1960); it contained nucleoproteins, a trace of residual lipid and all the tissue proteins except a small amount of proteolipid removed by the lipid solvents under these conditions. In some experiments the homogenized tissue suspension from 30 rat brains was made up to 250 ml with 0·25 M-sucrose and 4 ml portions were shaken with 4 ml of cold 2 N-H_2SO_4. The sediment obtained on centrifugation was then resuspended in 5 ml of N-H_2SO_4 for hydrolysis at 75°.

Hydrolysis of rat brain proteins. The dry protein preparation (40–80 mg) was weighed to within 0·05 mg in glass-stoppered test tubes and 5 ml of cold N-H_2SO_4 were added. The tubes were placed in a thermostatically controlled water bath at 100° or 75° and the contents were stirred. Tubes were removed at intervals and hydrolysis was stopped by rapid cooling to 0°. The tubes were then centrifuged at 1000 *g* and the supernatant was decanted. The sediment was resuspended in 5 ml of cold

N-H_2SO_4 and centrifuged again. The combined supernatants were made up to 10 ml and ammonia was estimated in four 1 ml portions.

Estimation of ammonia. Each 1 ml sample was added to 0·3 ml of 5 N-NaOH in the outer compartment of a Conway unit containing 0·4 ml of 0·05 N-HCl in the central compartment. After diffusion for 12 hr at 20° the acid solution was transferred to a test tube and the compartment was washed four times with 1 ml of distilled water. The acid solution and the combined washings were made up to 5 ml and 1 ml of Nessler reagent was added. The colour was read at 490 mμ and compared with that of standard solutions of ammonium sulphate which had been taken through the same procedure.

RESULTS

In preliminary experiments it was found that the total ammonia released from the protein fraction of rat brain on hydrolysis with N-H_2SO_4 for 2 hr at 100° corresponded to 527 μmoles amide-N/g dry protein (range of 510-549 μmoles in 4 experiments).

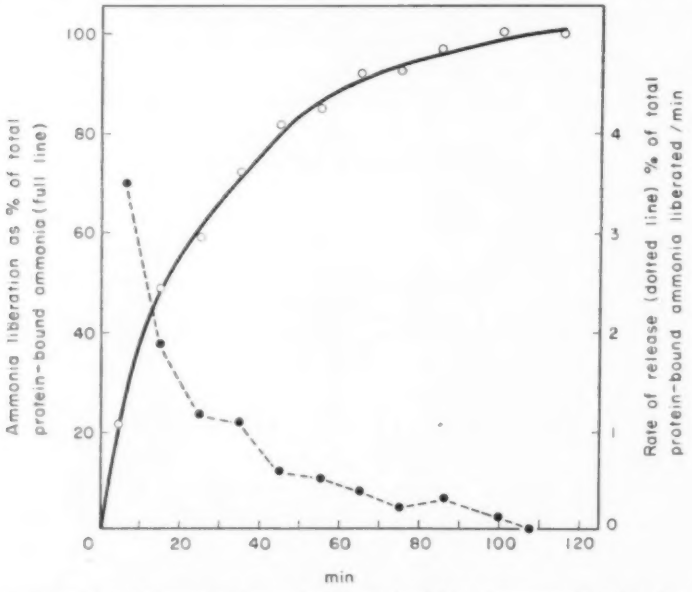

FIG. 1.—Liberation of ammonia from rat brain proteins during treatment with N-H_2SO_4 at 100°. (Each point is the mean of 2–4 experiments)

Under these conditions the hydrolysis of amide bonds appeared to be practically complete, as further hydrolysis yielded only small amounts of ammonia that might have come fron non-amide sources (Fig. 1).

On hydrolysis in N-H_2SO_4 at 100° the release of ammonia approximated to a continuous process and gave a relatively smooth curve. However, when hydrolysis of the same protein preparation was performed under milder conditions at 75° liberation of ammonia was clearly discontinuous; the hydrolysis of amide bonds appeared to proceed in a series of 'steps' (Fig. 2). The curve for the reaction rate indicated that the ammonia was liberated in 3 main bursts, with latent periods between them during which the rate of ammonia liberation was markedly decreased. After 5½ hr, when about 70-80 per cent of the total protein-bound ammonia had been released, there was a long latent period lasting for more than an hour when the rate of ammonia liberation fell almost to zero. These observations were repeated several times, using protein

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preparations from rats of different strains; in principle the curves were found to be reproducible. The deviation of the experimental curves from the theoretical curve for a continuous release of ammonia greatly exceeded the probable experimental error of the methods. It therefore appeared that some of the amide bonds of the rat brain protein preparation were relatively more stable towards acid hydrolysis under these conditions. This stable fraction could be clearly distinguished from the three more labile fractions liberated during the first $5\frac{1}{2}$ hr.

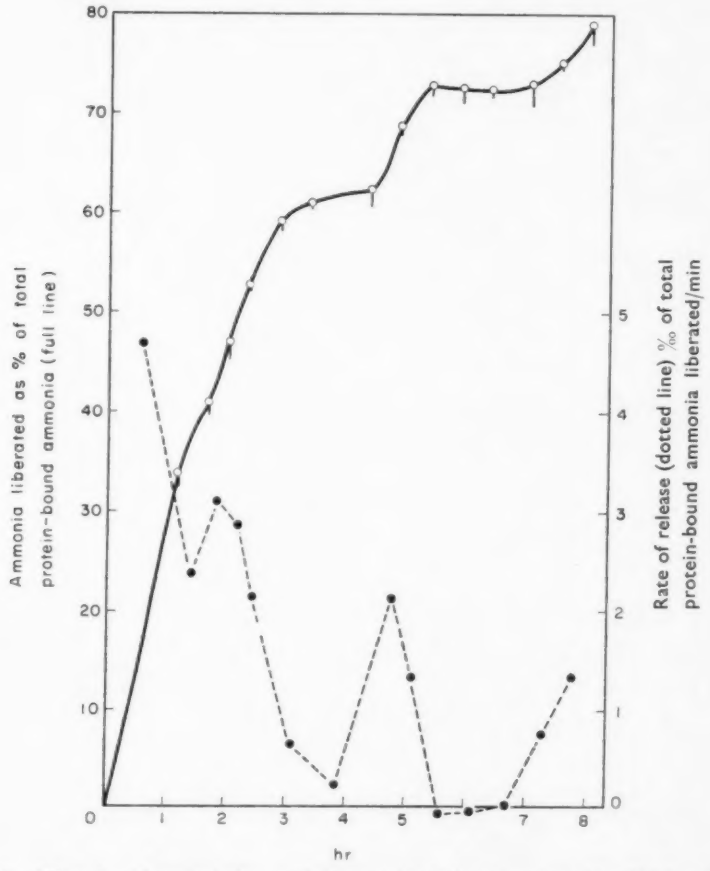

FIG. 2.—Liberation of ammonia from rat brain proteins during treatment with N-H_2SO_4 at 75°. (Each point is the mean of four experiments, vertical lines indicate S.D.).

It seemed possible that the discontinuous release of ammonia on hydrolysis might be an artifact produced by the trichloroacetic acid treatment, the lipid extraction, or the drying of the brain protein preparation. Experiments were therefore carried out to observe the release of ammonia during the hydrolysis of a homogenized suspension of fresh brain tissue which had not been treated with trichloroacetic acid or organic solvents. Curves showing a discontinuous liberation of ammonia were again obtained under these conditions (Fig. 3). The velocity curves showed similar depressions to those previously obtained and there was again evidence of a relatively stable residual

fraction remaining after about 75-80 per cent of the total protein-bound ammonia had been released.

The hydrolysis curve shown in Fig. 3, where measurements are given at 10 min intervals (instead of 30 min intervals as in Fig. 2), suggests that the 3 main labile fractions might be further sub-divided into smaller fractions. This was found also for

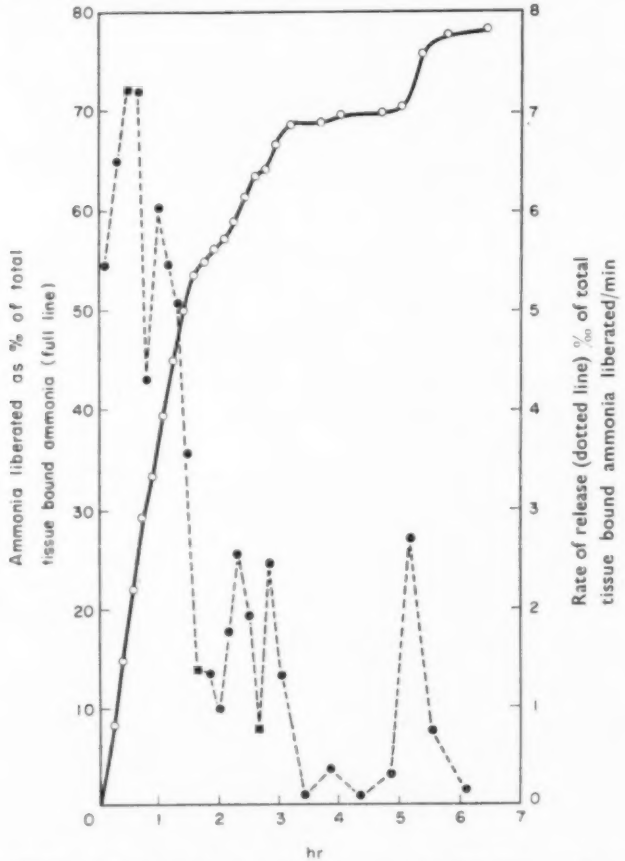

FIG. 3.—Liberation of ammonia from rat brain suspension during treatment with N-H_2SO_4 at 75°C.
(Each point is the mean of two experiments).

precipitated, extracted and dried rat brain protein preparations when the rate of ammonia liberation was followed at 8 min intervals. A discontinuous release of ammonia was found again in further experiments in which the hydrolysis was carried out with N-H_2SO_4 at 65°. Although decrease in the reaction temperature by 10° gave data which produced an extended hydrolysis curve, the peaks corresponding to the three main labile fractions and the more stable residue could still be seen.

In seeking an explanation for the discontinuity in ammonia liberation at 75° and 65°, protein samples were hydrolysed with N-H_2SO_4 at 75° for various periods in the 2-5 hr interval during which there is a well marked latent period. After hydrolysis for the periods indicated in Fig. 4, the supernatant fluid was separated by centrifugation

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from the protein residue and the ammonia content was determined. The supernatant fluid was then hydrolysed for a further 2 hr at 75°, after which the ammonia content was measured again. The data (Fig. 4, black circles) indicate that within the latent period further hydrolysis of the supernatant solution produced an additional amount of free ammonia. Part of the ammonia was therefore liberated directly from the insoluble protein, and part from a soluble hydrolysis product.

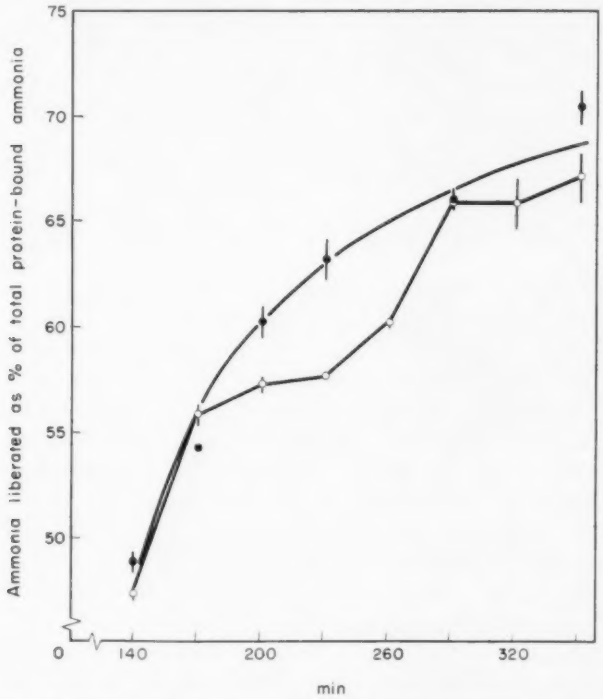

FIG. 4.—Liberation of ammonia from rat brain proteins during treatment with N-H_2SO_4 at 75°. The supernatant (N-H_2SO_4) was separated from the protein by centrifugation and decantation and ammonia was estimated (circles). Thereafter the rest of the supernatant was heated for an additional 2 hr at 75°, and analysed again for ammonia. (Full points).
(Each point is the mean of 4 experiments. Vertical lines indicate S.E.M.).

DISCUSSION

The present results obtained with brain proteins agree with previous observations on other proteins (OSBORNE and NOLAN, 1920; SHORE *et al.*, 1935-6) in showing that during hydrolysis in dilute acid at 100° the liberation of ammonia is practically a continuous process. On the other hand, under milder conditions (at 75° or 65°) the release of ammonia from rat brain proteins was not continuous. The observed 'steps' in the curves obtained for the release of ammonia from brain proteins during hydrolysis were not attributable to experimental error since (a) in principle they were reproducible and (b) they were not obtained when the hydrolysis was carried out at 100°. The discontinuity in the release of ammonia under mild conditions of hydrolysis was not connected with the experimental methods used in the preparation of the protein fraction, since similar curves were obtained for the release of ammonia from

homogenates of fresh brain tissue which had not been dried or treated with trichloroacetic acid, or lipid solvents.

Experiments carried out to ascertain the reasons for the discontinuous release of ammonia showed that part of the ammonia released during hydrolysis came from a soluble product which passed into solution during one of the latent periods. This suggests that the rate of hydrolysis of amide bonds is influenced by changes in the protein molecules produced by the partial hydrolysis of peptide bonds. It may be noted that 'stepped' curves, similar in appearance to those obtained in the present investigation, were previously observed by CHEESMAN and EHRENSVÄRD (1944) for the coupling of casein with dinitrophenylhydrazine during acid hydrolysis under conditions in which a partial hydrolysis of peptide bonds may be expected to take place. One possible explanation for the difference observed in the present experiments between the smooth hydrolysis curves obtained at 100°, and the 'stepped' curves at 75° could be that the hydrolysis of peptide bonds is a rate-limiting factor at lower temperatures, whereas at 100° it is relatively more rapid and the limiting factor is the rate of hydrolysis of amide bonds.

While part of the amide nitrogen of the rat brain proteins appears to be more labile in the sense that it is released more rapidly during mild acid hydrolysis, we are dealing with the hydrolysis of a mixture of proteins, which proceeds partly in solution and partly as a heterogeneous reaction. The conditions are clearly very complex and the apparent variation in lability might be explained in a number of different ways. It has been shown that the rate of hydrolysis of the amide groups of glutamine and asparagine combined in peptide linkage depends on the nature of neighbouring amino acid residues (THIERFELDER and VON CRAMM, 1919; MILLER and WAELSCH, 1952). The hydrolysis of amide groups may also be influenced by changes during hydrolysis in the spatial configuration of the protein molecules affecting the accessibility of amide groups to H^+ ions and water. Apart from steric factors of this kind, the hydrolysis of amide groups may be expected to be slowed down through increasing polar retardation as the reaction proceeds. Thus ARCUS (1949) showed that the release of ammonia from polymethacrylamide proceeds in 'steps' owing to the change in distribution of charges, and hence in the probability of hydrogen bonding between neighbouring amide and carboxyl groups, as the ratio of carboxyl to amide groups increases during hydrolysis.

Interest attaches to the amide-nitrogen of the brain proteins in view of the changes in protein-bound ammonia that have been reported to occur *in vivo* under physiological conditions (VRBA, 1955; GERSHENOVICH and KRICHEVSKAYA, 1960). There is also evidence that the amide bonds of proteins may be a site of attachment of pharmacologically active amines (BLOCK, 1947; CLARKE *et al.*, 1959). The present work suggests that in this connexion it might be useful to have further information about the distribution of amide groups in individual proteins in the brain under different physiological conditions.

SUMMARY

The release of ammonia from rat brain proteins during hydrolysis with N-H_2SO_4 at 100° approximates to a continuous process. Under milder conditions, at 75° and 65°, ammonia is released in steps.

The discontinuous release of ammonia during hydrolysis is not attributable to the treatment of the brain proteins with lipid solvents or to drying, since similar hydrolysis curves are obtained with homogenates of fresh brain tissue.

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THE *IN VITRO* INHIBITORY EFFECT OF LSD, ITS CONGENERS AND 5-HYDROXYTRYPTAMINE ON HUMAN CHOLINESTERASES*†

E. K. ZSIGMOND‡, F. F. FOLDES and V. M. FOLDES
Department of Anesthesia of the Mercy Hospital and the Section on Anesthesiology, Department of Surgery, The University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania

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ALTHOUGH it has been known since 1943 that D-lysergic acid diethylamide (LSD)§ is a potent psychotropic drug (STOLL and HOFMANN, 1943; STOLL, 1947), no satisfactory explanation has been offered for its mechanism of action (ROTHLIN, 1957*a*; ROTHLIN and CERLETTI, 1956). POLONI and MAFFEZZONI (1952) observed an increase of the acetylcholine (ACh) content in the brain of guinea pigs after the administration of LSD. Subsequently, THOMPSON, TICKNER and WEBSTER (1954) studied the effect of LSD on human cholinesterases, They reported that it reversibly inhibited human plasma cholinesterase (plasma ChE) and the pseudo cholinesterase of the human brain, but in concentrations which inhibited plasma ChE by 50 per cent, it had only slight effect on red cell ChE and the true cholinesterase of the brain. It was also reported by the same authors (THOMPSON *et al.*, 1955) that human cholinesterases were inhibited by LSD to a greater extent than cholinesterases of the rat, guinea pig, rabbit, chicken and monkey brain. TONINI (1955) found that rat brain cholinesterase activity was potentiated by 5-hydroxytryptamine (serotonin; 5-HT), LSD, and lysergic acid monoethylamide (LAE) in low concentrations. He, as well as FRIED and ANTOPOL (1956, 1957), who also noticed potentiation of human plasma ChE by LSD attempted to relate this effect to the psychotropic action of these compounds.

ZEHNDER and CERLETTI (1956) reported, however that the non-hallucinogenic 2-brom-D-lysergic acid diethylamide (BOL) (ROTHLIN and CERLETTI, 1952; CERLETTI and ROTHLIN, 1955) had an inhibitory effect on human plasma ChE similar to that of LSD. Although in recent years the psychotropic activity of many, newly synthetized derivatives of LSD was studied in man (SOLMS, 1953, 1956; ISBELL *et al.*, 1959; GIBERTI and GREGORETTI, 1958; GOGERTY and DILLE, 1957; CALLIERI, 1958; MURPHREE *et al.*, 1958; HOFMANN, 1959; SCHNECKLOTH *et al.*, 1957; ABRAMSON *et al.*, 1958) relatively little work has been done on the anticholinesterase effect of these compounds (GOGERTY and DILLE, 1957).

* Preliminary reports of this work were published in *Fed. Proc.* 1959 **18**, 463; and 1960 **19**, 266.

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‡ Present address: Allegheny General Hospital, Pittsburgh, Pennsylvania.

§ The following abbreviations are used: LSD, D-lysergic acid diethylamide bitartrate; 5-HT, 5-hydroxytryptamine creatinine sulphate; LAE, D-lysergic acid monoethylamide bimaleinate; BOL, 2-brom-D-lysergic acid diethylamide bitartrate; L-LSD, L-lysergic acid diethylamide bimaleinate; D-*iso*-LSD, D-*iso*-lysergic acid diethylamide bimaleinate; LSM, D-lysergic acid morpholide bimaleinate; MLD, D-1-methyl-lysergic acid diethylamide bitartrate; DAM, D-lysergic acid dimethylamide chlorhydrate; ACh, acetylcholine chloride; ATCh, acetylthiocholine iodide; BuCh, butyrylcholine iodide; BuTCh, butyrylthiocholine iodide; MeCh, acetyl-β-methylcholine chloride; BeCh, benzoylcholine chloride; ChE, cholinesterase.

The purpose of this study has been the investigation of the inhibitory effect of several psychotropic and non-psychotropic LSD derivatives on human cholinesterases. Because of the antagonistic effect of LSD on some of the pharmacological actions of 5-HT (GADDUM, 1953; CERLETTI and ROTHLIN, 1955; WOOLLEY and SHAW, 1954), a more detailed investigation of the earlier reported inhibitory effect of this compound on human cholinesterase (ERDÖS, BAART, SHANOR and FOLDES, 1956) was made.

MATERIAL AND METHODS

Materials

Source of enzymes. The source of plasma ChE was Cholase, a purified, concentrated human plasma cholinesterase preparation, prepared similarly to Harvard fraction IV-6-3. One ml of Cholase corresponds in activity to 160 ml of pooled, heparinized human plasma (FOLDES *et al.*, 1957).

Freshly obtained, heparinized, washed, and haemolysed human red cells, prepared according to AUGUSTINSSON (1949), were used as the source of red cell ChE.

Human grey matter ChE was obtained from the brain of adults who died suddenly (*e.g.*, rupture of aortic aneurysm, massive coronary occlusion, electrocution, etc.). Autopsies were performed within 5 hr of death. Before removing the brain, blood was washed out of it by perfusion with 0·9% NaCl. After this, the brains were sectioned, placed in plastic bags and quick-frozen by immersion in an acetone–dry ice mixture. The sections were stored below −20°. Subsequently, the grey matter of the cortex and the basal ganglia were separated from the white matter, homogenized in a glass homogenizer with 4 vol of a tissue bicarbonate buffer (NACHMANSOHN and ROTHENBERG, 1945). Homogenization was carried out at 4° and the homogenates were stored below −20°. Under these conditions, enzyme activity remained constant for at least 6 months.

Substrates. Acetylcholine chloride (ACh), acetylthiocholine iodide (ATCh), butyrylcholine iodide (BuCh), butyrylthiocholine iodide (BuTCh), benzoylcholine chloride (BeCh) and acetyl-β-methylcholine chloride (MeCh) were used.

The compounds studied were: D-Lysergic acid diethylamide bitartrate (LSD), L-lysergic acid diethylamide bimaleinate (L-LSD), D-*iso*-lysergic acid diethylamide bimaleinate (D-*iso*-LSD), 2-brom-D-lysergic acid diethylamide bitartrate (BOL), D-lysergic acid morpholide bimaleinate (LSM), D-1-methyl-lysergic acid diethylamide bitartrate (MLD), D-lysergic acid monoethylamide bimaleinate (LAE), D-lysergic acid dimethylamide chlorhydrate (DAM) and 5-hydroxytryptamine creatinine sulphate (5-HT). The structural formulae of the LSD derivatives are shown in Fig. 1.

The inhibitory effect of the breakdown products of LSD and BOL on human cholinesterases was also investigated. These were obtained by allowing LSD and BOL solutions, brought to pH 2·0 with HCl, to stand at room temperature, exposed to light for 7–10 days. The u.v. absorption curve of the breakdown product of LSD (Fig. 2), determined in a Beckman automatic recording spectrophotometer, is similar to that of lumilysergic acid A (HELLBERG, 1958).

Methods

The inhibitory effect of the compounds studied was measured with AMMON's (1934) modification of Warburg's manometric method at 37° and at pH 7·4. The total volume of the hydrolysates was 2 ml and the gas phase was 5% CO_2 : 95% N_2. All experiments were done in duplicate. Corrections were made for alkaline hydrolysis of the substrates, both in the presence and absence of the compounds tested. The compounds were preincubated with the enzymes for 15–20 min before the addition of the substrates. The inhibitory effect was observed with at least 6 different concentrations. Three of the concentrations were above and 3 below the I_{50} values, which were determined by plotting the negative logarithms of the inhibitor concentrations against activity. The data presented are the means obtained from at least three experiments. The substrate concentration of ACh with Cholase was 22·0 mM, with red cell ChE and grey matter ChE it was 3·1 mM. Preliminary studies showed that the latter concentration was optimal for the activity of human grey matter ChE. The substrate concentrations of BuCh, MeCh and BeCh were 22·0 mM with all enzymes. ATCh and BuTCh were used in the same concentrations as their oxy-analogues. The final Cholase concentration was 1:8000 (v/v) for all substrates; that of red cells was 1:165 (v/v) for ACh and 1:55 (v/v) for MeCh and that of human grey matter homogenates was 1:75, 1:50 and 1:25 (w/v) for ACh, MeCh and BuCh, respectively. With Cholase 0·025M-$NaHCO_3$ buffer was used; with red cells and brain grey matter homogenate,

the buffers described by NACHMANSOHN and ROTHENBERG (1945) were used. Experimental conditions with the breakdown products of LSD and BOL were the same as described above. The effect of changing the pH between 6·0 and 8·0 on the LSD induced inhibition of the hydrolysis of BeCh by Cholase was determined by a modification of KALOW's (1952) u.v. spectrophotometric method in a Beckman model DU spectrophotometer. The BeCh concentration in these experiments was 0·05 mM

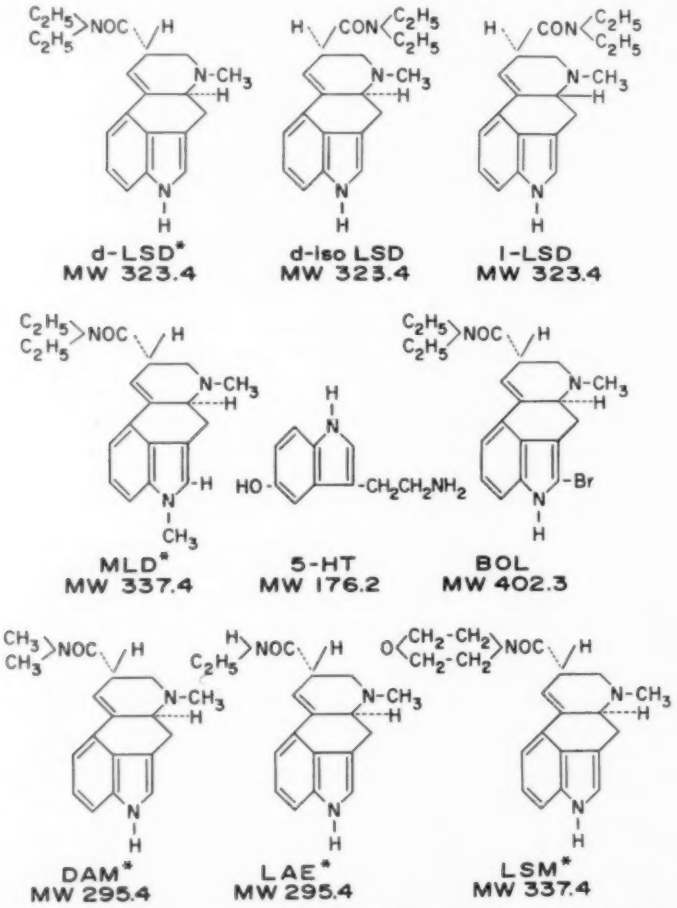

FIG. 1.—The structural formulae of the compounds tested. The compounds marked with an asterisk have hallucinogenic activity.

and that of Cholase was 1:30,000 (v/v). Readings were taken at 30 sec intervals until 50 per cent of the substrate was hydrolysed. SÖRENSEN's (1909) phosphate buffers were used in these experiments. The temperature was kept constant at 37°. The total volume of the system was 4 ml. The light path was 10 mm. In a few experiments, pooled, heparinized human plasma was used instead of Cholase.

RESULTS

The inhibitory effect of the compounds tested on the hydrolysis of ACh, BuCh and BeCh by plasma ChE is summarized in Table 1. There is no relationship between the inhibitory effect of the compounds tested on the activity of plasma ChE and their psychotropic activity. Although the inhibitory effect of D-LSD, the most potent

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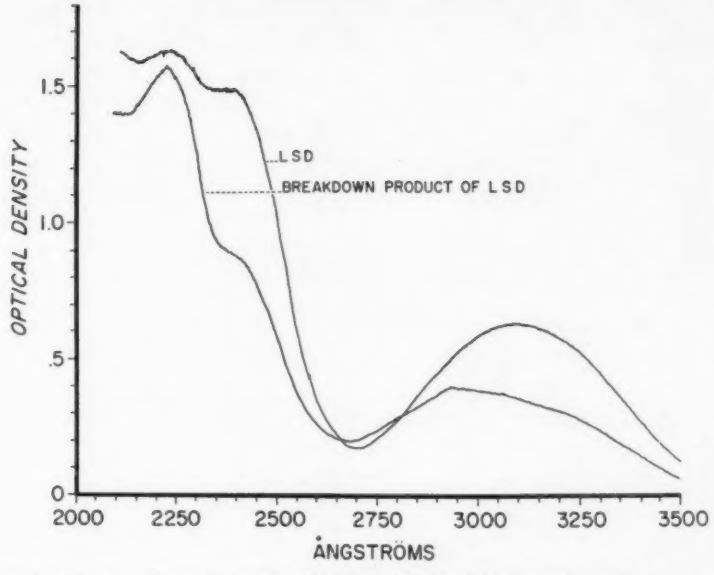

FIG. 2.—The u.v. absorption curves of LSD and its breakdown product. The concentration of the compounds was 0·033 mM.

TABLE 1.—THE INHIBITORY EFFECT OF LYSERGIC ACID DERIVATIVES AND 5-HT ON HUMAN PLASMA CHOLINESTERASE

Compounds	I_{50} values (M)		
	ACh $115{\cdot}5^{*} \pm 5{\cdot}1$	BuCh $232{\cdot}3 \pm 21{\cdot}1$	BeCh $36{\cdot}2 \pm 3{\cdot}6$
Hallucinogenic			
D-LSD	$1{\cdot}4 \times 10^{-6}$	$3{\cdot}3 \times 10^{-6}$	$5{\cdot}3 \times 10^{-6}$
MLD	$6{\cdot}7 \times 10^{-5}$	—	—
LAE	$1{\cdot}5 \times 10^{-4}$	—	—
DAM	$2{\cdot}8 \times 10^{-5}$	—	—
LSM	$1{\cdot}0 \times 10^{-4}$	$6{\cdot}0 \times 10^{-3}$	Insignificant
Non-hallucinogenic			
L-LSD	$1{\cdot}0 \times 10^{-5}$	—	—
D-*iso*-LSD	$7{\cdot}6 \times 10^{-6}$	—	—
BOL	$4{\cdot}9 \times 10^{-6}$	$1{\cdot}6 \times 10^{-5}$	$9{\cdot}7 \times 10^{-5}$
5-HT	$9{\cdot}4 \times 10^{-4}$	$2{\cdot}6 \times 10^{-3}$	Insignificant

* Uninhibited rate: μmoles hydrolysed by 1 ml of plasma in 30 min ± S.E.M. Values are means of at least 3 experiments.

hallucinogenic compound, is the greatest, L-LSD, D-*iso*-LSD (ROTHLIN, 1957*a*, *b*) and BOL (CERLETTI and ROTHLIN, 1955), which are psychotropically inactive, inhibit this enzyme to a greater extent than the psychotropically active MLD (ISBELL *et al*, 1959), LAE (ROTHLIN, 1957*a*), DAM (ISBELL *et al.*, 1959), and LSM (GOGERTY and DILLE, 1957). Comparison of the structural formulae of the compounds (Fig. 1) with their I_{50} values (Table 1) indicates that substitution of both ethyl radicals on the amino

nitrogen with methyl groups as in DAM, or one with a hydrogen, as in LAE, or the enclosure of the two ethyl groups into a morpholine ring, as in LSM, or the substitution of a methyl group on the indole nitrogen decreased the inhibitory effect of the compounds on plasma ChE. Stereo-isomeric changes, as in L-LSD and D-*iso*-LSD, also decreased the inhibitory effect on plasma ChE.

Similarly, no correlation could be detected between the antiserotonin activity of the compounds (CERLETTI and ROTHLIN, 1955; CERLETTI and DOEPFNER, 1958; and

TABLE 2.—THE INHIBITORY EFFECT OF LYSERGIC ACID DERIVATIVES AND 5-HT ON HUMAN RED CELL CHOLINESTERASE

Compounds	I_{50} values (M)	
	ACh 302·4* ± 3·5	MeCh 59·4 ± 4·9
Hallucinogenic		
D-LSD	$4·9 \times 10^{-5}$	$9·0 \times 10^{-5}$
MLD	$7·1 \times 10^{-6}$	—
LAE	$4·3 \times 10^{-3}$	—
DAM	$4·3 \times 10^{-4}$	—
LSM	$1·5 \times 10^{-3}$	$2·4 \times 10^{-3}$
Non-hallucinogenic		
L-LSD	$2·0 \times 10^{-3}$	—
D-*iso*-LSD	$4·2 \times 10^{-4}$	—
BOL	$6·8 \times 10^{-5}$	$1·0 \times 10^{-4}$
5-HT	$4·3 \times 10^{-3}$	$5·4 \times 10^{-3}$

* Uninhibited rate: μmoles hydrolysed by 1 ml of red cells in 30 min ± S.E.M. Values are means of at least 3 experiments.

ISBELL *et al.*, 1959) and their anti-plasma ChE effect. Furthermore, there was little difference between the inhibitory effect of 5-HT and that of some of its antagonists, *e.g.* MLD and DAM (ISBELL *et al.*, 1959), on the hydrolysis of plasma ChE.

The inhibitory effect of the compounds on the hydrolysis of ACh and MeCH by red cell ChE is summarized in Table 2. With the exception of MLD, the inhibitory effect of all compounds tested was lower for red cell ChE than for plasma ChE, and the relative potency of the compounds for the two enzymes was about the same. MLD, however, had a greater inhibitory effect on red cell ChE than on plasma ChE.

The inhibitory effect of the compounds on the hydrolysis of ACh and MeCH by human grey matter ChE was about the same as that on the hydrolysis of these substrates by human red cell ChE. Similarly, the inhibitory effect of the compounds on the hydrolysis of BuCh by human grey ChE and by human plasma ChE were of the same order of magnitude. This is in line with the finding that most of the human grey matter ChE is true cholinesterase (NACHMANSOHN and ROTHENBERG, 1945).

The results with ATCh and BuTCh substrates were similar to those obtained with their oxy-analogues.

The breakdown products of LSD and BOL inhibited the hydrolysis of various substrates by human plasma ChE and red cell ChE to about the same extent as their parent compounds.

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LSD, in concentrations ranging from 10^{-11} to 10^{-8}M, neither accelerated nor inhibited the activity of the enzymes tested.

Changing the pH of the reaction medium from 6·0 to 7·0 increased three-fold the inhibitory effect of LSD on the hydrolysis of BeCh by plasma ChE. Further increase of the pH from 7·0 to 8·0 caused no change in inhibitory potency. Since the p*K*b value of LSD is 7·12 (FISHER, 1956), it seems that there is no close relationship between the degree of ionization and the anticholinesterase activity of LSD.

TABLE 3.—THE INHIBITORY EFFECT OF LYSERGIC ACID DERIVATIVES AND 5-HT ON HUMAN GREY MATTER CHOLINESTERASE

Compounds	I_{50} values (M)		
	ACh 164·9* ± 10·1	MeCh 83·0 ± 5·2	BuCh 21·6 ± 0·6
Hallucinogenic			
D-LSD	$6{\cdot}3 \times 10^{-5}$	$1{\cdot}1 \times 10^{-4}$	$2{\cdot}5 \times 10^{-6}$
MLD	$2{\cdot}8 \times 10^{-5}$	—	—
LAE	—	$1{\cdot}0 \times 10^{-3}$	$3{\cdot}3 \times 10^{-3}$
DAM	$3{\cdot}8 \times 10^{-4}$	—	—
LSM	$3{\cdot}1 \times 10^{-3}$	$6{\cdot}5 \times 10^{-3}$	$1{\cdot}4 \times 10^{-3}$
Non-hallucinogenic			
L-LSD	—	$4{\cdot}1 \times 10^{-3}$	$1{\cdot}0 \times 10^{-4}$
D-*iso*-LSD	—	$3{\cdot}0 \times 10^{-4}$	$9{\cdot}0 \times 10^{-5}$
BOL	$3{\cdot}0 \times 10^{-5}$	$1{\cdot}8 \times 10^{-4}$	$1{\cdot}6 \times 10^{-5}$
5-HT	$7{\cdot}6 \times 10^{-3}$	$9{\cdot}8 \times 10^{-3}$	$8{\cdot}4 \times 10^{-3}$

* Uninhibited rate: μmoles hydrolysed by 1 g of wet tissue in 30 min ± S.E.M. Values are means of at least 3 experiments.

5-HT was a weak inhibitor of the hydrolysis of all substrates by the three enzymes tested. Its inhibitory effect was additive with that of LSD.

The results of the experiments with freshly obtained, pooled, human plasma were the same as those obtained with Cholase.

DISCUSSION

The findings presented indicate that LSD and some of its congeners have a significant inhibitory effect on both true and pseudo-cholinesterases. This finding is in contradistinction to those of THOMPSON *et al.* (1955), who reported that LSD only inhibited plasma ChE and had no significant inhibitory effect on human red cell and brain cholinesterases. The discrepancy between the findings of THOMPSON *et al.* (1955) and those of ours may be explained by the fact that the substrate concentration used by them was considerably higher (15 mM) than the concentration (3·1 mM) found to be optimal in our study. The 0·049 mM-LSD concentration which gave 50 per cent inhibition with the substrate concentration used in this work only gave a 15 per cent inhibition with the concentration used by THOMPSON (ZSIGMOND *et al.*, 1959).

Except for MLD, all compounds investigated inhibited human plasma ChE to a greater extent than human red cell ChE or grey matter ChE. In agreement with

THOMPSON *et al.* (1955), rabbit cholinesterases were inhibited less than human cholinesterases by LSD derivatives in equivalent concentrations (ZSIGMOND *et al.* Unpublished data).

Plotting the reciprocal of the inhibited and uninhibited rate ($1/v$) against the reciprocal of the substrate concentration ($1/S$) according to LINEWEAVER and BURK (1934) resulted in straight lines intersecting the ordinate at the same point both with plasma ChE (Fig. 3) and red cell ChE (Fig. 4). This indicates that LSD is a true

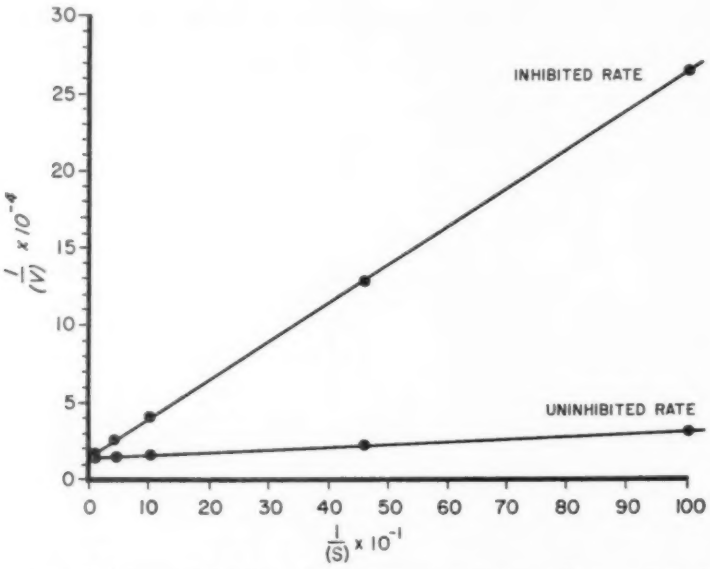

FIG. 3.—The influence of changing substrate concentration on the inhibitory effect of LSD on the hydrolysis of ACh by plasma cholinesterase. The figure indicates competitive inhibition.

competitive inhibitor of the hydrolysis of ACh by both enzymes. Probably the other structurally closely-related compounds studied are also competitive inhibitors.

Although ATCh and BuTCh are hydrolysed about 30 per cent faster than the corresponding oxy-esters, LSD inhibited their hydrolysis by cholinesterases to the same extent as that of their oxy-analogues. In contrast to the findings of TONINI (1955) and FRIED and ANTOPOL (1957), it could not be demonstrated that LSD in low concentrations accelerated the hydrolysis of ACh by human plasma ChE.

No correlation was found between the *in vitro* inhibitory effect and the hallucinogenic activity of the compounds tested. BOL, L-LSD and D-*iso*-LSD, compounds without any significant hallucinogenic effect (CERLETTI and ROTHLIN, 1955; ISBELL *et al.*, 1959; MURPHREE *et al.*, 1958; ROTHLIN, 1957*a*), inhibited cholinesterases to about the same extent as the highly hallucinogenic LSD. On the other hand, MLD, LAE, DAM and LSM, which are hallucinogenic, had less inhibitory effect on plasma ChE than the non-hallucinogenic compounds. Although the inhibitory effect of the hallucinogenic MLD on red cell ChE and grey matter ChE was the greatest, the more strongly hallucinogenic LSD inhibited these enzymes to the same extent as the non-hallucinogenic BOL. The breakdown products of LSD (see Fig. 2) and BOL which

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have no psychogenic effects (ROTHLIN, 1957*b*) also inhibited cholinesterases to the same extent as the mother compounds.

No correlation could be demonstrated between the anti-serotonin and the anti-cholinesterase activity of the compounds tested. There was no major difference in the anticholinesterase effect of LSD and BOL which are potent antagonists of 5-HT (GADDUM, 1953; CERLETTI and ROTHLIN, 1955) and that of L-LSD and D-*iso*-LSD which did not inhibit 5-HT (ROTHLIN 1957*a*; CERLETTI and DOEPFNER, 1958). Furthermore, the anticholinesterase effect of 5-HT and that of its pharmacological antagonists D-LSD and BOL, were found to be additive.

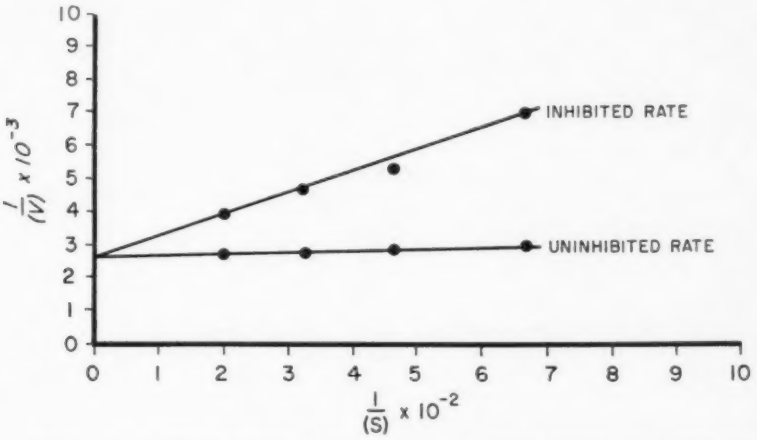

FIG. 4.—The influence of changing substrate concentration on the inhibitory effect of LSD on the hydrolysis of ACh by red cell cholinesterase. The figure indicates competitive inhibition.

Comparison of the structural formulae and the anticholinesterase activity of the compounds tested suggests the possible significance of ethyl substitution on the amino nitrogen. Those compounds in which two ethyl groups are attached to the amino nitrogen, D-LSD, MLD, L-LSD, D-*iso*-LSD and BOL, were more potent inhibitors of both true and psuedo-cholesterases than LAE where one of the ethyl groups is substituted with hydrogen, or DAM in which both ethyl groups are substituted by methyl radicals, or LSM in which both ethyl groups were incorporated in a morpholine ring. Substitution of a methyl group on the indole nitrogen of LSD significantly decreased the inhibitory effect of the resulting compound, MLD, on plasma ChE and slightly increased its inhibitory effect on red cell ChE and grey matter ChE.

SUMMARY

The inhibitory effect of eight LSD derivatives and of 5-hydroxytryptamine on human plasma, red cell and brain grey matter cholinesterases was investigated. With the exception of MLD, plasma cholinesterase was inhibited more by all compounds tested than red cell or grey matter cholinesterases. No correlation was found between the *in vitro* anticholinesterase activity and the hallucinogenic or anti-serotonin activity of the compounds tested.

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The determination of the u.v. absorbtion curve of LSD and its breakdown product was carried out by Dr. E. G. ERDÖS, Division of Fundamental Research, Mellon Institute, Pittsburgh, Pa.

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QUANTITATIVE HISTOCHEMICAL STUDIES OF THE MORPHOGENESIS OF THE CEREBELLUM

I—TOTAL LIPID AND FOUR ENZYMES*

ELI ROBINS and IRENE P. LOWE†
Department of Psychiatry and Neurology, Washington University School of Medicine, St. Louis, Missouri

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THE HISTOLOGICAL appearance of the layers of the developing cerebellar cortex in the albino rat has been described in detail by ADDISON (1911). In the foetal rat, as late as the nineteenth day, only the presumptive cerebellar layers—ependymal, mantle, and marginal—can be readily distinguished. At birth, or just before, relatively defined layers appear. These are the external granular layer, the molecular layer (including the Purkinje cells at its inner margin), the internal granular layer, and the subjacent white matter. Once these 4 layers have appeared (the white matter immediately subjacent to the internal granular layer will be referred to as a layer in this report), it is possible to follow discretely each into adult life or until disappearance (external granular layer).

The early segregation of the layers of the cerebellar cortex offered, therefore, an unusual opportunity to study the development in the central nervous system of relatively homogeneous histological regions. To understand the chemistry of the developing nervous system in detail, it will be necessary ultimately to study it in terms of its individual histological elements. While this observation may be true for any tissue, it is particularly applicable to the nervous system because of its extreme architectural complexity.

Because of the minute size of these individual cerebellar layers, their quantitative chemical study required the use of sensitive microchemical procedures and their isolation, under direct histological control, in a chemically intact state (LOWRY, 1953; ROBINS, 1957). The use of the rat offered an advantage, since almost the entire development of the cerebellum occurs postnatally.

In this report, we will describe changes in total lipid, lactic dehydrogenase (LDH)‡, malic dehydrogenase (MDH), isocitric dehydrogenase (ICDH), and dipeptidase (PEPT) in the individual cerebellar layers of the 0-day (newborn), 9-day, 14-day, and adult rat. These quantitative histochemical data will be supplemented with data from

* Supported by a grant from the United Cerebral Palsy Association.

† Present address: Department of Anatomy, University of Minnesota School of Medicine, Minneapolis, Minn.

‡ Abbreviations used are: LDH, lactic dehydrogenase; MDH, malic dehydrogenase; ICDH, isocitric dehydrogenase; PEPT, dipeptidase (unpublished data indicate that the peptidase being measured is probably a dipeptidase); TRIS, trishydroxyaminomethane·HCl buffer; DPN, DPNH, oxidized and reduced forms of diphosphopyridine nucleotide, respectively; TPN, TPNH, oxidized and reduced forms of triphosphopyridine nucleotide, respectively.

the whole cerebellum (for protein and total fresh weight) and from whole brain homogenates (for the four enzymes) at 0, 4, 9, 12, 14, 21, and 200 or more days of age.

The choice of the four ages for obtaining the histochemical data was determined by the studies of ADDISON (1911) on the developing cerebellar cortex, of WATSON (1903) and TILNEY (1933) on myelination in the cerebellum, and of JACOBS and SNIDER (1949) on the development of electrical activity in the cerebellum.

TABLE 1.—BRIEF DESCRIPTION OF HISTOLOGIC CHANGES IN THE DEVELOPING CEREBELLUM OF THE RAT

Layer	Age in Days		
	0–9	9–14	14–Adult
External granular	Increasing thickness, reaching peak at day-10.	At day-10 begins to decrease in thickness, chiefly by cell migration, and is less than half its maximal thickness by day-14.	Disappears about day-21.
Molecular	Little visible change.	At day-8 this layer begins to widen owing to arborizations of the processes of the Purkinje cells. From day-8 to -11 Nissl substance becomes apparent, horizontal cells appear, and basket cells begin to differentiate. By day-14 the layer is much wider owing to the continuing arborizations of the processes of the Purkinje cells, which have formed a single layer at the base.	Reaches adult appearance by about day-25, except for further arborizations of the Purkinje cell processes.
Internal granular	Little change up to day-5. From day-5 to day-9 the numbers of granule cells begin to increase slowly.	At day-9 rate of increase of granule cells becomes markedly accelerated and continues throughout this period. At day-12 grouping into glomeruli begins and is distinct by day-14. Axons of mossy and climbing fibres begin to appear near the end of this period.	By day-17 the layer is much thicker and more cellular. Subsequently, the adult form is gradually assumed.
White matter	Just visible signs of myelination are present by 7–8th day.	At day-9 slight myelination is present. By day-14 there has been a tremendous increase in number of fibres and degree of myelination.	Myelination continues until about day-40.

Newborn and adult animals were chosen in order to have a baseline and a final picture, respectively. The choice of the 9-day and 14-day animals was dictated by both histological and physiological considerations. The histological changes in each layer are most rapid and profound from the 9th to 14th day (Table 1). The physiological changes reach their culmination in the middle of this period, at day 12, when electrical activity characteristic of the adult cerebellum, 10–60 μv with a frequency of 150–250 cyc/sec, first appears. It is possible, therefore, by choosing 9 and 14 days postnatally, to study the chemistry just before and after these significant histological and physiological changes occur.

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MATERIAL AND METHODS

Animals. Pregnant albino rats of the Holtzman strain were received at about the nineteenth day of pregnancy. The young rats were born within 90 hr of arrival. At the appropriate times they were decapitated, and the brain was removed within 2 min. The mothers were used as adult controls and were killed 1–2 weeks after their young were weaned.

Quantitative histochemistry. The general procedure for obtaining unthawed, unfixed, unstained and histologically defined material has been described (LOWRY, 1953). The cerebellum, still attached to the midbrain and medulla-pons in the 0-day and 9-day animals, was frozen in liquid nitrogen and sectioned at −20° in a cryostat; at these ages, the entire block (cerebellum and brain stem) was sectioned because of the small size of the cerebellum. Because of the fragility of the tissue in the newborn animals, it was difficult to use a brush to lead the microtome sections off the knife (LOWRY, 1953), and a device similar to that described by LINDERSTRØM-LANG and MOGENSEN (1938) was used.

TABLE 2.—CONDITIONS AND VOLUMES FOR ASSAY OF THE FOUR ENZYMES

The numbers in parentheses are concentrations during incubation in m-moles per litre. Enzymic incubation for all enzymes was 30 min at 38°.

Conditions	Enzymes			
Assay Conditions	LDH	MDH	ICDH*	PEPT
pH	7·45	8·7	8·2	7·5
Buffer	Tris (100)	Tris (100)	Tris (80)	$NaPO_4$ (100)
Substrate	pyruvate (0·8)	oxalacetate (1)	isocitrate (5)	glycyl-L-phenylalanine (10)
Pyridine nucleotide	DPNH (1·25)	DPNH (1·25)	TPN (0·25)	—
Nicotinamide	(20)	—	(20)	—
Bovine serum albumin (%)	0·05	0·05	0·05	0·02
Volumes				
During incubation (μl)	2·5	5	1	1
During reading (ml)	1·1	1·1	0·06	1
Fluorescence measurement				
Exciting light, mμ†	365	365	365	365
Emitted light, mμ	460–480‡	460–480‡	460–480‡	450–470§

* $MnCl_2$ (0·2 mM) was also present during incubation.
† Obtained with Corning glass filter No. 5860.
‡ Obtained with Corning glass filters Nos. 5562, 4308, and 3387, in combination.
§ Obtained with Corning glass filters Nos. 5543 and 3387, in combination.

The tissue fragments to be analysed weighed 0·008–0·113 μg. It was necessary to use such small pieces in the 0-day animal in order to dissect cleanly the individual layers. In the older animals it would have been possible to obtain larger fragments, but to make the analyses comparable the same size pieces were used. The pieces were weighed on a 'fishpole' type quartz fibre balance (LOWRY, 1953). The lipid-free dry weight was determined after extraction with alcohol and hexane (LOWRY, 1953).

Chemical methods. (Table 2). The measurement of LDH, MDH and ICDH was based on the fluorometric measurement of the pyridine nucleotide produced during the enzymic reaction (LOWRY, ROBERTS and KAPPHAHN, 1957; LOWRY, ROBERTS and CHANG, 1956). For LDH and MDH, after incubation an equal volume of 0·44 N–HCl was added to destroy excess DPNH present in the substrate. To four-fifths of the sample were added 100 μl of 6·4 N–NaOH (for LDH) or of 7·0 N –NaOH (for MDH). After incubating at 38° for 30 min to convert the enzymically produced DPN to a fluorescent product, 1 ml of water was added, and the fluorescence was measured in a Farrand fluorometer, model A. For the measurement of ICDH, after the enzymic incubation, 2·4 μl of 0·08 N –NaOH were added, and the tubes incubated for 60 min at 38° in order to destroy the excess TPN

present in the substrate. Ten μl of 8·2 N–NaOH which was 0·01 per cent in hydrogen peroxide were added to oxidize simultaneously the TPNH and convert the resulting TPN to a fluorescent product. A 10 μl portion was transferred to a special microfluorometer tube (LOWRY, ROBERTS, LEINER, WU and FARR, 1954) containing 50 μl of water and read in the fluorometer. The measurement of PEPT was based on the fluorometric measurement of the free phenylalanine produced by enzymic action from glycyl-L-phenylalanine (Table 2). After the enzymic incubation, 2 μl of 32 mM-ninhydrin in 0·5 M-phosphate buffer, pH = 6·8, were added, and the resulting mixture was heated for 60 min at 60°. A 2·5 μl portion was removed and placed in a tube containing 1 ml of 0·3 M-$NaHCO_3$. The fluorescence was determined in the Farrand fluorometer. The details of this method will be published later. Protein was determined colorimetrically (LOWRY, ROSEBROUGH, FARR, and RANDALL, 1951).

Studies on cerebellar homogenates. To supplement the quantitative histochemical data on the developing cerebellum, the weights and protein content of the whole cerebellum were determined in siblings of the animals used for the histochemical studies. The weights of the whole developing cerebellum were measured after dissecting the entire cerebellum from the brain. The cerebellum was homogenized in water for subsequent protein measurement.

Studies on whole brain homogenates. The remainder of each brain (the cerebellum having been used in the preparation of the cerebellar homogenates) rostral to the caudal border of the inferior colliculus was homogenized in 0·01 M-Tris (pH = 7·5) and analysed for the four enzymes and for protein content. These enzymes were measured in homogenates to obtain a picture of the enzymic changes in whole brain and to ascertain the amount of variation among animals, since the histochemical data to be presented are from only one animal at each time. The second paper in this series (ROBINS, FISHER and LOWE, 1961) will present histochemical data on two animals at each time for two other enzymes, and will present additional evidence that there is relatively slight variation between animals.

Statistical methods. Means and standard errors were calculated by the usual methods (OSTLE, 1954). The standard errors were calculated on the basis of data from one animal and include, therefore, biological and measurement variability. Tests of significance were *t*-tests. A significance level of 0·01 was accepted as being significantly different.

TABLE 3.—FRESH WEIGHT OF THE DEVELOPING CEREBELLUM

Age of animals (days)	No. of animals	Weight of cerebellum* (mg)	per cent compared to 0-day	Daily increases in weight (mg/day)
0	5	9·0	100	
4	5	26·9	300	4·47
9	3	81·3	904	10·9
12	2	126·5	1410	15·0
14	2	144·6	1610	9·05
21	2	187·7	2090	6·15
Adult	2	251·2	2810	0·40†

* Two families of animals were used, family F and family G. The values in this column are the means. The individual values were: 0-day, F: 10·4, 11·0, 11·1, G: 6·1, 6·3; 4-day, F: 26·7, 26·9, 27·2, G: 26·4, 27·2; 9-day, F: 85·6, 83·0, G: 75·3; 12-day, F: 124·3, G: 128·7; 14-day, F: 141·7, G: 147·5; 21-day, F: 190·5, G: 184·8; Adult, F: 243·3, G: 259·1.

† Assuming adult age is 180 days.

RESULTS

Weight and protein content of developing whole cerebellum. The entire cerebellum increased almost 30-fold in weight during development (Table 3). The maximal rate of increase was from day-9 to day-12, but the rate was high from day-4 to day-14, and fell precipitately sometime after day-21. The agreement between siblings and between families was excellent except at day-0 where the cerebellums of family G weighed less than those of family F.

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The amount of protein in the entire cerebellum increased almost 60-fold during development (Table 4). The percentage of protein approximately doubled during development. The highest rate of absolute increase in protein content was from day-12 to day-14, but the rate was appreciably high from day-4 to day-21. The greatest increase in percentage of protein per day occurred from day-12 to day-14. It is evident from the findings concerning both the weight and the protein content of the developing cerebellum that day-9 to day-14 is a period of extremely active development.

TABLE 4.—PROTEIN CONTENT OF THE DEVELOPING CEREBELLUM*

Age of animals (days)	No. of animals	Protein (mg)	Daily increase in protein (mg/day)	Per cent protein† (% fresh weight)
0	2	0·48		5·37
4	4	1·72	0·31	6·38
9	3	6·24	0·90	7·69
12	2	8·99	0·92	7·14
14	2	12·0	1·50	8·32
21	2	18·5	0·93	9·86
Adult	2	27·6	0·06‡	11·0

* The protein content in this table is very similar to that reported by CLOUET and GAITONDE (1956) in the developing rat cerebellum at 7, 8, 17, and 58–84 days.

† The values in this column are the means. The results for the individual animals from families F and G were: 0-day, F: 5·58, G: 5·16; 4-day, F: 6·55, 6·37, G: 6·58, 6·00; 9-day, F: 8·37, 7·82, G: 6·86; 12-day, F: 7·18, G: 7·10; 14-day, F: 8·41, G: 8·23; 21-day, F: 10·05, G: 9·67; Adult, F: 10·4, G: 11·7.

‡ Assuming adult age is 180 days.

Enzymic activities in brain homogenates. (Fig. 1). Little change occurred from 0 to 4 days in LDH, MDH, or PEPT. From 4 days to adulthood each of these enzymes increased, more or less in parallel. MDH increased the most, but this was only a 2-fold increase. LDH and PEPT increased only 70 to 80 per cent during development. The results for MDH and LDH are similar to those previously reported in developing whole rat brain (KUHLMAN and LOWRY, 1956). (The higher absolute levels of the two enzymes in the present study were found because the enzymes were measured in the faster direction, *i.e.* reduction of the substrate.) The rate of increase between 9 and 14 days for each of these enzymes was not particularly high when compared with other periods.

ICDH showed a strikingly different pattern from the other three enzymes in the whole brain homogenates. ICDH decreased and in the adult was less than one-quarter of its 0-day value. There was a decrease from day-0 to day-4, little change from day-4 to day-9, and a further decrease from day-9 into adulthood.

Amount of lipid in layers of the developing cerebellum. (Table 5). The lipid in the external granular layer showed only a slight increase, although statistically significant, from 0 to 14 days. The molecular layer showed a progressive increase in lipid, relative to the lipid-free dry weight, with increasing age, amounting to a 2·5-fold increase by adult life. The increase from 0 to 9 days was not statistically significant, but from day-9 onward the increases were significant ($P < 0{\cdot}01$). The internal granular layer showed no significant change at any age. The subjacent white matter showed a significant increase at all times from 0 days to adulthood. These findings demonstrate that

there is a large amount of lipid deposited in the molecular layer and white matter during development, but that there is no net deposit of lipid in the internal granular layer, and only a relatively slight deposit in the external granular layer.

Enzymic activities in the layers of the developing cerebellum. The results (Tables 6–9) are presented on the basis of total dry weight. Since there is a significant amount of

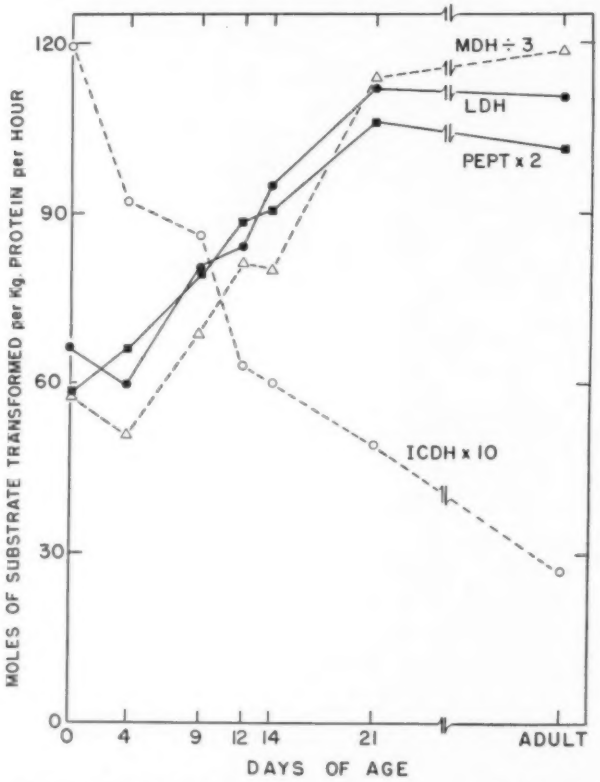

FIG. 1.—Changes in activities of 4 enzymes of whole rat brain during postnatal development. The plotted values are the means from 2(12, 14, 21 day, and adult), 3(4 and 9 day) or 5(0-day) animals at each age for each enzyme. In each instance, values from 2 families were obtained. The agreement between siblings and between families was satisfactory. The minimal and maximal values of individual animals for each enzyme at each age follow. LDH: 0-day (58·0, 68·7); 4-day (54·4, 65·7); 9-day (77·6, 83·4); 12-day (79·4, 89·4); 14-day (91·5, 99·0); 21-day (108, 117); adult (109, 110). MDH: 0(133, 205); 4(149, 157); 9(196, 225); 12(234, 252); 14(238, 242); 21(337, 347); adult (354, 359). ICDH: 0(10·8, 13·0); 4(8·33, 10·0); 9(8·48, 8·76); 12(6·04, 6·52); 14(5·95, 6·06); 21(4·78, 4·93); adult (2·66, 2·75). PEPT: 0(24·6, 30·2); 4(29·7, 38·0); 9(35·1, 44·8); 12(42·7, 45·6); 14(44·6, 45·4); 21(52·5, 54·0); adult (50·2, 51·5). The percentage of protein (means) increased as follows: 0(6·29%), 4(6·60%), 9(7·19%), 12(8·28%), 14(9·24%), 21(9·53%), adult (10·9%).

lipid deposited in the molecular and white matter layers during development, the results in Figs. 2–5 have been calculated on the basis of lipid-free dry weight, so that comparisons between animals of different ages can be made independent of the amount of (enzymically inactive) lipid deposited.

LDH (Fig. 2) in the external granular layer decreased and was about one-half its 0-day value at 9 and 14 days. In the molecular layer, LDH increased slightly at 14

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TABLE 5.—LIPID PER KG LIPID-FREE DRY WEIGHT IN THE LAYERS OF THE DEVELOPING CEREBELLUM

All values are expressed as g lipid per kg lipid-free dry weight. Each value is the mean of 4 to 10 measurements ± S.E.M.

Age (days)	Layers of the cerebellum			
	External granular	Molecular	Internal granular	White matter
0	180 ± 8	200 ± 12	252 ± 11	290 ± 5
9	227 ± 17	245 ± 13	222 ± 18	492 ± 15
14	233 ± 7	327 ± 23	260 ± 15	895 ± 30
Adult		509 ± 10	276 ± 9	1695 ± 27

TABLE 6.—LDH IN THE LAYERS OF THE DEVELOPING CEREBELLUM

All values are expressed as moles of substrate transformed per kg dry weight per hr. Each value is the mean of 4 to 8 determinations on one animal ± S.E.M.

Age of rat (days)	Layers			
	External granular	Molecular	Internal granular	White matter
0	44·7 ± 3·7	28·8 ± 2·7	25·0 ± 1·7	32·2 ± 2·6
9	20·1 ± 2·5	27·4 ± 0·8	23·8 ± 1·7	30·2 ± 2·6
14	24·5 ± 2·0	28·3 ± 1·5	28·9 ± 0·7	36·1 ± 0·9
Adult		38·7 ± 1·3	37·0 ± 1·7	20·2 ± 1·0

TABLE 7.—MDH IN THE LAYERS OF THE DEVELOPING CEREBELLUM

All values are expressed as moles of substrate transformed per kg dry weight per hr. Each value is the mean of 4 to 8 determinations on one animal ± S.E.M.

Age of rat (days)	Layers			
	External granular	Molecular	Internal granular	White matter
0	70·6 ± 6·5	81·9 ± 5·9	96·8 ± 9·7	78·5 ± 7·6
9	80·0 ± 6·8	120 ± 11	118 ± 12	129 ± 8
14	84·0 ± 8·3	143 ± 14	124 ± 7	115 ± 15
Adult		284 ± 10	215 ± 17	119 ± 8

TABLE 8.—ICDH IN THE LAYERS OF THE DEVELOPING CEREBELLUM

All values are expressed as moles of substrate transformed per kg dry weight per hr. Each value is the mean of 4 to 8 determinations on one animal ± S.E.M.

Age of rat (days)	Layers			
	External granular	Molecular	Internal granular	White matter
0	2·51 ± 0·17	2·65 ± 0·14	2·43 ± 0·16	2·89 ± 0·11
9	2·51 ± 0·06	3·04 ± 0·16	2·58 ± 0·13	3·13 ± 0·16
14	3·41 ± 0·18	3·10 ± 0·16	3·13 ± 0·22	2·69 ± 0·23
Adult		1·13 ± 0·13	1·51 ± 0·04	0·89 ± 0·02

TABLE 9.—PEPT IN THE LAYERS OF THE DEVELOPING CEREBELLUM

All values are expressed as moles of substrate transformed per kg dry weight per hr. Each value is the mean of 4 to 8 determinations on one animal ± S.E.M.

Age of rat (days)	Layers			
	External granular	Molecular	Internal granular	White matter
0	4·72 ± 0·09	7·72 ± 0·50	6·17 ± 0·33	8·25 ± 0·45
9	13·0 ± 0·7	16·4 ± 0·4	15·1 ± 1·9	24·3 ± 1·5
14	12·4 ± 0·5	13·5 ± 1·4	14·4 ± 1·0	20·7 ± 0·7
Adult		18·5 ± 0·9	12·1 ± 0·9	9·09 ± 0·80

days, but its activity almost doubled between 14 days and adulthood. The changes in the internal granular layer were similar to those in the molecular layer. Changes in white matter contrasted with those in the other layers. There was a significant increase of 50 per cent from day-9 to day-14, and a significant decrease from 14 days to adulthood, although the final adult level was higher than at 0 and 9 days.

MDH (Fig. 3) in the external granular layer showed negligible change at 9 and 14 days. In the molecular layer there was a significant increase of 52 per cent at 9 days, and a further increase, especially marked after 14 days, till in the adult it was more than

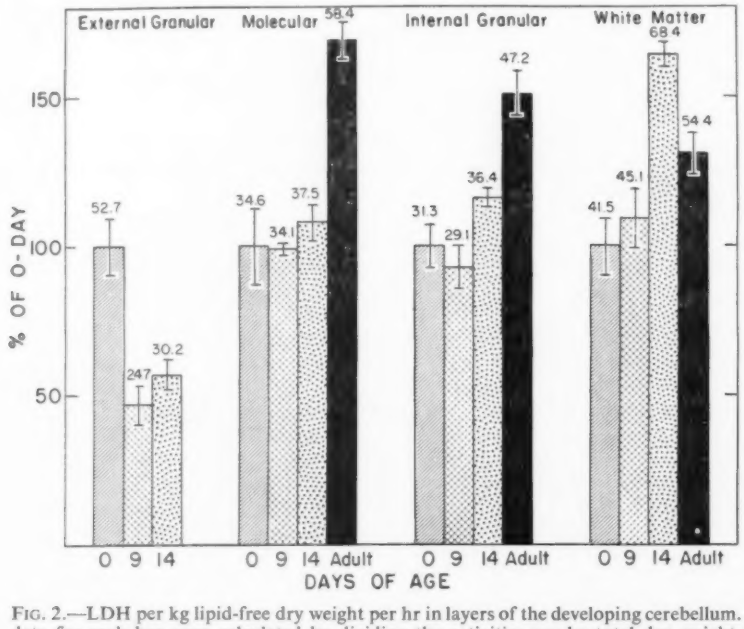

FIG. 2.—LDH per kg lipid-free dry weight per hr in layers of the developing cerebellum. The data for each bar were calculated by dividing the activities per kg total dry weight (from Table 6) by the corresponding lipid-free dry weight per kg total dry weight (calculated from Table 5) for each layer at each age. (The g lipid-free dry weight per kg total dry weight may be calculated from the values in Table 5 as in the following example: Molecular layer at 14 days = 327 g lipid per kg lipid-free dry weight. $327 + 1000 = 1327$. $\frac{1000}{1327} = 754$ g lipid-free dry weight per kg total dry weight.) The figures at the top of each bar are activities, expressed as moles of substrate transformed per kg lipid-free dry weight per hr. The lines with the cross bar at each end indicate ± S.E.M.

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4 times the 0-day level. Changes in the internal granular layer followed the same pattern as those in the molecular layer but were only half as great. Changes in white matter too were similar in pattern, with a somewhat greater increase up to 9 days, and an overall 3-fold increase in the adult instead of 4-fold as in the molecular layer.

ICDH (Fig. 4) showed slight increases in all layers up to 14 days. From 14 days to adulthood there was a marked and significant decrease in all layers to less than half

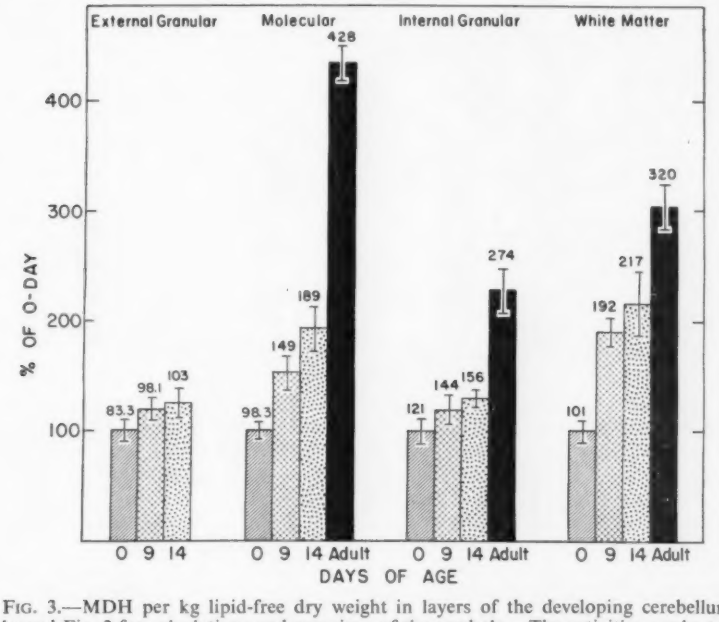

FIG. 3.—MDH per kg lipid-free dry weight in layers of the developing cerebellum. See legend Fig. 2 for calculations and meanings of the symbols. The activities per kg total dry weight per hr used in the calculations are from Table 7.

the maximum values. The final levels were only 60 per cent of those in the newborn animals. This decrease for ICDH was in striking contrast to the other 3 enzymes. It was qualitatively similar, however, to the decrease found in the whole brain homogenates (Fig. 1).

PEPT (Fig. 5) showed at least a 2–3 fold increase between 0 and 9 days in all layers. There was little change between 9 and 14 days in any of the layers. Between 14 days and adulthood there was a further significant increase of PEPT in the molecular layer, but a slight decrease in the internal granular layer. In the white matter between 14 days and adulthood there was a significant decrease in PEPT, but the final level was still significantly higher than the levels at 0 and 9 days.

DISCUSSION

For the whole cerebellum the period from 9 to 14 days is one of an extremely high rate of cerebellar growth and protein deposition (Tables 3 and 4). These high rates of growth for the whole cerebellum do not indicate, however, the differences among individual layers . The increased information available from a quantitative histochemical analysis of such histologically defined regions as the individual cerebellar layers is apparent with regard to lipid deposition. The differential rate of lipid deposition, as

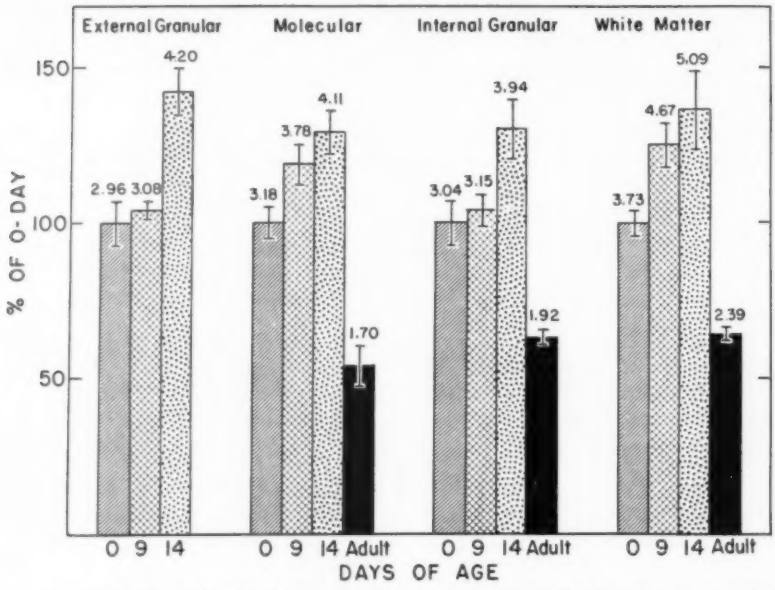

FIG. 4.—ICDH per kg lipid-free dry weight per hr in layers of the developing cerebellum. See legend Fig. 2 for calculations and meanings of the symbols. The activities per kg total dry weight per hr used in the calculations are from Table 8.

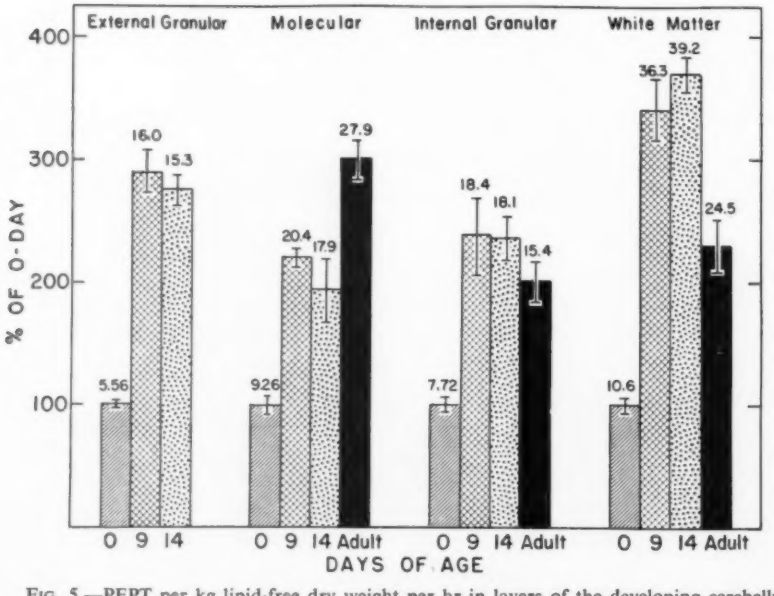

FIG. 5.—PEPT per kg lipid-free dry weight per hr in layers of the developing cerebellum. See legend Fig. 2 for calculations and meanings of the symbols. The activities per kg total dry weight per hr used in the calculations are from Table 9.

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measured by the increase in lipid deposited relative to the lipid-free dry weight, varies markedly among the layers. The amount of lipid in the internal granular layer did not change significantly at any period during development, whereas that in the molecular layer and subjacent white matter did (Table 5). The greatest daily rate of differential deposition occurred in both of these layers between 9 and 14 days. This rate amounted to over 6 per cent per day for the molecular layer and to over 16 per cent per day for the white matter.*

The layer-by-layer analysis indicates, therefore, that: (1) In the internal granular layer there is no net excess of lipid deposited, so that the lipid-free dry weight of this layer remains virtually constant throughout development. (2) In the molecular layer, even though this is also a grey layer, a significant amount of excess lipid is deposited. (3) In the white matter, the excess deposition of lipid follows classical lines, which reflect the process of myelination. (4) In the layers where excess lipid is deposited, the rate of deposition is greatest from 9 to 14 days.

These findings strongly suggest that there are important differences in lipid metabolism between synaptic (molecular) and cell-body rich (internal granular) regions of the central nervous system. It also seems apparent that myelinated areas (subjacent white matter) have a different lipid metabolism than regions of either kind of grey matter.

These findings concerning lipid deposition raise at least two questions. First, does the absence of an excess of lipid deposition in the granular layer signify that the nature of the lipids present remains unchanged with age, or that, if their composition changes, it is in a direction different from that of the molecular layer and white matter? The differences in the mole fraction of non-phosphorus-containing sphingolipids in the three layers in the adult cerebellum (ROBINS, EYDT and SMITH, 1956) suggest that one or other of these alternatives is probably correct and that a different series of events occurs in each layer of the developing cerebellar cortex during the period of lipid deposition. Second, is the excess of lipid deposited in the molecular layer by *in situ* synthesis, brought by the blood? The first possibility suggests the presence of certain enzymes or substrates in the molecular layer not present in the granular layer, while the second raises the problem as to why these lipids are not deposited in the internal granular layer as well.

The rapid rate of protein and lipid deposition from 9 to 14 days, the profound histological changes during this period (Table 1) and the first appearance of adult electrical activity from the cerebellum at day-12 (JACOBS and SNIDER, 1949), all suggest that the period of 9 to 14 days postnatally may correspond for the rat cerebellum to the 'critical period' described by FLEXNER and his associates for the cerebral cortex in both the guinea pig and the rat. These workers recorded an increase in lipid in the foetal guinea pig at 41 to 45 days of gestation (FLEXNER J. B. and FLEXNER L. B., 1950; FLEXNER L. B. and FLEXNER J. B., 1950), and at the same time marked histological

* As an example of the calculation of these rates, the computation for white matter from 9 to 14 days will be given (Table 5).

$$\frac{895-492}{492} \times 100 = 82\% \text{ increase in lipid per kg lipid-free dry weight}$$

$$\frac{82}{14-9} = 16{\cdot}4\% \text{ increase in lipid per kg lipid-free dry weight per day}$$

The rates from 14 days to adulthood were calculated on a 26-day basis, as SPERRY (1955) showed that net lipid deposition in rat brain virtually ceases at 40 days after birth.

changes (PETERS and FLEXNER, 1950), the onset of cortical electrical activity (FLEXNER, TYLER and GALLANT, 1950), and the beginning of responses to electrical stimulation of the cerebral cortex (KIMEL and KAVALER, 1951). Similar data for the rat cerebral cortex have been obtained for the histological changes (SUGITA, 1917), and for the first appearance of electroencephalographic activity similar to that in the adult (CRAIN, 1952). This period in the rat cerebral cortex apparently appears at about 10 days postnatally.

The enzymic activities reported in this study might be expected, at least in some instances, to show changes coincident with the assumed critical period occurring between 9 and 14 days. The results already presented show, however, that only LDH might be said to fit this expected pattern. The other three enzymes did not show a change beginning at 9 days and could not be said to identify a 'critical period'. The present results suggest at least two possibilities: (1) The 'critical period' defined by FLEXNER and his associates in studies of the guinea pig and rat is critical for specified enzymes with certain (unknown) biological functions, whereas for others either a critical period does not exist or occurs at a different time; (2) The cerebral and cerebellar cortices are different in respect of the significance of enzyme changes during a 'critical period'.

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LDH in the molecular and internal granular layers remains essentially unchanged up to the 9th day, begins to increase from day-9 to -14, and continues to increase into adult life. The increase from day-9 to day-14 in the molecular layer is slight, however, and not statistically significant. In white matter, there is also an increase from day-9 to day-14, but a significant decrease from day-14 to adulthood, which differs from the patterns of the molecular and internal granular layers. These findings suggest that glycolysis, if LDH is representative, may be involved in the 'critical period' of the developing rat cerebellum. This suggestion gains some support from the findings of FLEXNER and his associates concerning aldolase in the cerebral cortex of both the guinea pig (FLEXNER, GREENBLATT, COOPERBAND and FLEXNER, 1956) and the rat (HAMBURGH and FLEXNER, 1957). These workers showed an increase of aldolase in the rat cerebral cortex beginning at the tenth day postnatally and in the guinea pig at 35 days after conception, just prior to the critical period in this species.

The finding of a significant decrease of LDH in white matter from day-14 to adulthood contrasts with the further increase observed in the molecular and internal granular layers. Since this comparison is based on lipid-free dry weight, it is a specific activity and indicates that there is an actual relative decrease in enzymically active protein in the adult white matter. It is tentatively suggested that LDH is related to glial differentiation or myelin deposition as well as to general glycolytic metabolism in the growing animal, and that in the adult animal the two former functions have diminished or disappeared, and only the general metabolic function is quantitatively retained.

For MDH, in contrast to LDH, increases in activity for each layer begin at birth and continue into adult life. Although there are quantitative differences among the layers, the general pattern is similar for all. This finding suggests that there is no specific function for MDH during development as there appears to be for LDH.

MDH and LDH have been measured in developing cerebral cortex of the rat at 0 and 10 days and in adulthood by KUHLMAN and LOWRY (1956). Comparisons may be made between the corresponding molecular layers of the cerebral and cerebellar

cortices and between the subjacent white matter of each. For LDH in the rat cerebral cortex, when the newborn value is set at 100, the values in the molecular layer are 100, 84, and 131 and in white matter 100, 123, and 123 at 0 days, 10 days, and adulthood, respectively. These values agree well (Fig. 2) with those in the homologous layers of the cerebellar cortex at corresponding times. This suggests that changes in LDH, and perhaps in glycolytic metabolism generally, do not differ appreciably during the development of the cerebral and cerebellar cortices.

The MDH values in the rat cerebral cortex during development, when the newborn value is set at 100, are for the molecular layer 100, 113, and 173, and for the white matter 100, 82, and 36 at 0 days, 10 days, and adulthood, respectively (KUHLMAN and LOWRY, 1956). Changes in the molecular layers of both cortices (Fig. 3) are identical in direction but differ in magnitude, being approximately four times as great in the cerebellar as in the cerebral molecular layer. The results in white matter, however, show a different pattern. In the cerebral cortex there is a continuing decrease to one-third of the newborn value, whereas in the cerebellar cortex there is a 3-fold increase into adulthood. The differing patterns of change in MDH suggest that malate metabolism, and perhaps citric acid cycle metabolism generally, differ markedly in the cerebellar and cerebral white matter. Since the results for MDH on whole brain homogenates in the present study are similar to those of KUHLMAN and LOWRY (1956), the differences cannot be ascribed to differences of method. The differences may be real or may result from the fact that only one animal at each time was studied in each investigation. The latter explanation seems unlikely, however, since the histochemical pattern of the enzymic changes was consistent within each study at each time, increasing or decreasing, as the case may be.

ICDH, even though a member of the same metabolic cycle as MDH, showed a very different pattern from MDH during development, both in the whole brain homogenates and in the cerebellar layers. There were slight increases between 0 and 14 days but a marked decrease between 14 days and adulthood. The higher levels during development suggest a specialized function for ICDH in the developing brain and cerebellum, common to all histological layers. The observation of KUHLMAN and LOWRY (1956) that glucose-6-phosphate dehydrogenase decreased in the adult and the present finding concerning ICDH suggest that the higher levels of these enzymes in the developing brain are necessary to produce more TPNH for the synthetic processes (HORECKER and HIATT, 1958) occurring during that period. When these processes are over or greatly diminished, as in the adult, less TPNH is needed and these enzymes decrease.

PEPT differs from the other three enzymes already discussed in that it exhibits its most marked increases in all layers from day-0 to day-9. Changes from day-9 to day-14 are insignificant. There is a further significant increase in PEPT in the molecular layer in the adult, but this latter increase is less than that occurring from day-0 to day-9. In contrast, levels of PEPT in internal granular and white matter layers in the adult show no change or a significant decrease.

SUMMARY

The weight and protein content of the whole cerebellum of the rat was determined at birth, in adulthood, and at five intervening times. The cerebellum showed the

greatest rate of increase in wet weight and in protein content between 9 and 14 days postnatally.

Total lipid, lactic dehydrogenase, malic dehydrogenase, isocitric dehydrogenase, and dipeptidase were measured in the individual layers of the developing cerebellar cortex and in the immediately subjacent white matter at birth, in adulthood, and at two intervening times.

The total lipid measurements showed that there was a significant deposition of lipid, relative to the amounts of protein deposited, in the molecular layer and white matter during development. In contrast, in the internal granular layer there was no net deposition of lipid, relative to the amount of protein deposited, at any time during development.

The rates of deposition of lipid in the molecular layer and white matter were highest from 9 to 14 days postnatally.

The rapid rate of protein and lipid deposition from 9 to 14 days postnatally, the marked histological changes during this same period, and the first appearance of adult electrical activity at 12 days postnatally all suggested that the period 9 to 14 days postnatally may correspond for the rat cerebellum to the 'critical period' described by FLEXNER and his associates for the cerebral cortex in the guinea pig and rat.

The findings for the four enzymes measured, with the possible exception of lactic dehydrogenase, did not suggest that the period of 9 to 14 days was a critical period. Such a period, therefore, has not been defined for the rat cerebellum.

Isocitric dehydrogenase and dipeptidase showed patterns which departed from those of the other two enzymes. This departure was most striking for isocitric dehydrogenase which showed a significantly higher level in the developing cerebellum than in the adult cerebellum; this result suggests a special role for isocitric dehydrogenase in cerebellar development.

In addition to quantitative differences for each enzyme among the different layers, there were two instances where the enzymes in white matter behaved differently from that of the grey layers. Lactic dehydrogenase in white matter from day-14 to adulthood decreased, whereas in the molecular and internal granular layers it increased. Dipeptidase in white matter from day-14 to adulthood decreased, whereas in the molecular layer it increased and in the granular layer it did not change significantly.

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QUANTITATIVE HISTOCHEMICAL STUDIES OF THE MORPHOGENESIS OF THE CEREBELLUM

II. TWO β-GLYCOSIDASES*

ELI ROBINS, H. KENNETH FISHER† and IRENE P. LOWE‡
Department of Psychiatry and Neurology, Washington University School of Medicine, St. Louis, Missouri

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IN THE preceding paper (ROBINS and LOWE, 1961), the changes in malic, lactic, and isocitric dehydrogenases, in dipeptidase, and in total lipid of the individual layers of the developing cerebellar cortex of the rat were described. In the present study changes in two β-glycosidases—β-glucuronidase and β-galactosidase—will be described. These enzymes have been shown to increase greatly in peripheral nerves (HOLLINGER and ROSSITER, 1952; MCCAMAN and ROBINS, 1959) and in central nerves (MCCAMAN and ROBINS, 1959) undergoing Wallerian degeneration. It was of interest, therefore, to ascertain the changes in these enzymes in developing cerebellar white matter as well as in layers of the cerebellar cortex.

MATERIAL AND METHODS

The animals (rats) used and the methods of obtaining the frozen-dried, unfixed and unembedded cerebellar tissue and of measuring lipid-free dry weight have been described or referred to in Part I (ROBINS and LOWE, 1961). The preparation of the brain homogenates is also described in that paper.

The method for measuring the enzymic activities was a quantitative microchemical fluorimetric method, based on a modification of the procedure originally described by MEAD, SMITH and WILLIAMS (1955). The method depends on the release, by enzymic activity, of free 4-methylumbelliferone from the corresponding 4-methylumbelliferone glycoside. The free algycon is highly fluorescent, whereas in its glycosidic linkage it has only a negligible fluorescence. This sensitive procedure permitted the use of the small bits of tissue necessary to obtain discrete pieces from each layer of the cerebellum in the 0-day (newborn) animal. The samples varied in weight from 0·015 to 0·115 μg (usually 0·015 to 0·060 μg). To make all analyses comparable, samples of the same size were obtained from the older animals, although it would have been possible to obtain larger samples in this case.

The substrate solution for the β-galactosidase assay is prepared just before use by mixing 150 μl of 1·3 mM-4-methylumbelliferone-β-D-galactoside (generously supplied by Dr. MELVIN COHN), 5 μl of 4 M-sodium acetate buffer, pH 3·75 (after dilution to 0·12 M), 8 μl of 1 M-$MgCl_2$, and 2 μl of 5% bovine serum albumin. Final concentrations were 1·2 mM-4-methylumbelliferone-β-D-galactoside, 0·12 M-acetate buffer, 50 mM-$MgCl_2$, and 0·05% bovine serum albumin. The substrate solution for the β-glucuronidase assay is prepared just before use by mixing 150 μl water, 6 μl of 4 M-sodium acetate buffer, pH 3·75 (after dilution to 0·1 M), 75 μl of 2·1 mM-4-methylumbelliferone-β-D-glucuronide (generously supplied by Professor R. T. WILLIAMS), and 2·5 μl of 5% bovine serum albumin. Final concentrations of reagents were 0·1 M-acetate buffer, 0·7 mM-4-methylumbelliferone-β-D-glucuronide, and 0·05% bovine serum albumin. (All solutions used were prepared in glass-distilled water and preserved at −20°. All are indefinitely stable except for the 4-methylumbelliferone standard solution, which should be checked once a month.) The remainder of the analysis is identical for the two enzymes. To the frozen-dried sample in a special pointed Pyrex tube of 1·8 mm bore

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† Work done during the tenure of a Medical Student Research Fellowship of the United States Public Health Service.
‡ Present address: Department of Anatomy, University of Minnesota School of Medicine, Minneapolis, Minnesota.

(LOWRY, ROBERTS and CHANG, 1956) was added without mixing, 1 μl of the appropriate substrate solution at room temperature, and each tube was placed in an ice bath. The tubes were capped with Parafilm and incubated in a water bath for 2 hr at 38°. After the incubation 20 μl of 0·1 M-2-amino-2-methyl-1-propanol HCl buffer pH 10·4, were added to each tube with mixing. A 17 μl sample was removed and placed in a special microfluorometer tube (LOWRY, ROBERTS, LEINER, WU and FARR, 1954) which had already received 45 μl of the same pH 10·4 buffer. Readings were taken in a Farrand fluorometer, model A, adapted to receive the microfluorometer tubes (LOWRY *et al.*, 1954). A Corning glass filter, No. 5860, is used in the primary to isolate the 365 mμ Hg line, and a combination of filters, Nos. 5543 and 3387, is used in the secondary to isolate the emitted fluorescence between 450 mμ and 470 mμ. Blanks, standards, and control brain homogenates were included in triplicate in each analysis. The blanks were 1 μl of substrate solution added to empty tubes. The standards and control homogenates were prepared in the appropriate substrate solution and 1 μl of each of these mixtures was added to an empty tube. The 1 μl of homogenate solution contained 0·4 μg of brain, fresh weight, and the 1 μl of standard solution contained 5×10^{-11} mole of 4-methylumbelliferone.

Statistical comparisons were made of the changes with age for each enzyme in each layer. Since data were obtained from two animals at each time (Tables 1 and 2), it was desirable to combine the results from each pair of animals in order to draw Figs. 2 and 3 and in order to make statistical comparisons. The pooling and comparison of the data from each pair of animals were done in four steps (OSTLE, 1954): (1) The homogeneity of the variances of each animal of the pair was tested. (2) If the variances were homogeneous, a *t*-test was applied to ascertain if the animals differed significantly. (3) If they did not differ, the data from the two animals were pooled into a single mean with its own newly calculated standard error. (4) The significance of differences among data from a given layer at different ages was tested by means of the *t*-test. A significance level of 0·01 or lower was accepted as being statistically significant. In only two instances, β-galactosidase in the external granular layer of the newborn and β-galactosidase in the molecular layer of the 9-day animals, was there a significant discrepancy between the two animals, so that statistical pooling could not be done.

RESULTS

Enzymic activities in brain homogenates. As in Part I (ROBINS and LOWE, 1961) a whole brain homogenate was prepared from the brain rostral to the caudal border of the inferior colliculi and, therefore, excluded the caudal brain stem and cerebellum. In whole brain homogenates (Fig. 1) β-galactosidase activity was higher than β-glucuronidase activity at birth and remained so throughout development.

β-Galactosidase showed only negligible changes throughout development. Activity was slightly less in the adult than in the newborn (Fig. 1). β-Glucuronidase showed only slight changes up to the 14th day. At day-21 and in adulthood β-glucuronidase showed about 30 and 55 per cent increases, respectively, when compared with the newborn.

Enzymic activities in the layers of the developing cerebellum. β-Glucuronidase activities in the two animals at each time for each layer showed good agreement (Table 1). The maximal discrepancy between two animals at any age in one layer was 37 per cent, and that occurred in the internal granular layer of the newborn animals. The odd-numbered rats were all from the same family. There was no significant tendency for the developmental curves of the related rats of family A to be more alike than those for the rats from families B, C, and D. β-Galactosidase activities in the two rats at each time for each layer also showed good agreement, with two exceptions (Table 2). There was approximately a 2-fold discrepancy between rats 1 and 2 in the external granular layer, and between rats 3 and 4 in the molecular layer. For the 30 possible comparisons between animals for the two enzymes (each layer at each age) there were, therefore, only two significant discrepancies in enzyme levels.

TABLE 1.—β-GLUCURONIDASE IN DEVELOPING CEREBELLAR CORTEX OF THE RAT

Units are m-moles of substrate hydrolysed per kg dry brain ± S.E.M. The numbers in parentheses indicate the number of measurements.

Age in days	Rat family	Rat No.	Layers of cerebellum			
			External granular	Molecular	Internal granular	White matter
0	A	1	37·1 ± 4·8 (10)	25·9 ± 1·2 (5)	34·9 ± 2·7 (6)	29·6 ± 3·6 (11)
	C	2	40·1 ± 4·7 (6)	23·8 ± 3·7 (6)	21·9 ± 1·3 (5)	23·7 ± 3·1 (6)
9	A	3	29·4 ± 3·4 (6)	20·4 ± 3·9 (6)	43·6 ± 2·8 (6)	62·8 ± 10·2 (6)
	B	4	30·3 ± 3·4 (6)	29·8 ± 2·7 (6)	36·1 ± 3·2 (6)	72·8 ± 6·7 (6)
14	A	5	12·7 ± 2·4 (6)	10·2 ± 1·7 (5)	19·2 ± 1·3 (6)	25·7 ± 5·5 (6)
	B	6	16·0 ± 2·0 (6)	12·8 ± 1·8 (5)	25·6 ± 1·5 (5)	31·2 ± 3·4 (6)
Adult	A	7	—	9·8 ± 1·5 (6)	15·5 ± 2·2 (6)	4·1 ± 0·7 (5)
	D	8	—	7·5 ± 2·1 (5)	15·0 ± 1·5 (6)	2·7 ± 0·6 (6)

TABLE 2.—β-GALACTOSIDASE IN DEVELOPING CEREBELLAR CORTEX OF THE RAT

Units are m-moles of substrate hydrolysed per kg dry brain ± S.E.M. The numbers in parentheses indicate the number of measurements.

Age in days	Rat family	Rat No.	Layers of cerebellum			
			External granular	Molecular	Internal granular	White matter
0	A	1	44·8 ± 6·0 (6)	45·0 ± 4·6 (5)	36·2 ± 6·9 (6)	41·7 ± 4·0 (5)
	C	2	80·2 ± 10·0 (10)	34·5 ± 6·3 (5)	27·1 ± 1·3 (6)	26·5 ± 4·9 (6)
9	A	3	34·1 ± 5·7 (6)	30·6 ± 3·1 (6)	48·1 ± 3·4 (6)	101 ± 6·6 (5)
	B	4	34·2 ± 5·3 (8)	73·1 ± 6·0 (25)	62·7 ± 8·1 (6)	77·9 ± 6·3 (5)
14	A	5	46·9 ± 6·4 (6)	47·6 ± 5·6 (6)	59·2 ± 2·4 (6)	86·0 ± 7·6 (6)
	B	6	51·0 ± 7·2 (5)	52·2 ± 7·2 (6)	64·8 ± 9·9 (6)	75·3 ± 3·5 (6)
Adult	A	7	—	28·3 ± 5·0 (6)	77·3 ± 12·8 (6)	10·0 ± 2·3 (5)
	D	8	—	26·2 ± 2·1 (6)	99·1 ± 8·4 (6)	10·7 ± 1·7 (6)

The results (Tables 1 and 2) are presented on the basis of total dry weight. Since there is a significant amount of lipid deposited in the molecular and white matter layers during development (see Table 5, ROBINS and LOWE, 1961), the results in Figs. 1 and 2 have been calculated on the basis of lipid-free dry weight, so that comparisons among animals of different ages can be made independent of the amount of (enzymically inactive) lipid deposited.

Between birth and 9 days β-glucuronidase (Fig. 2) showed slight and insignificant changes in the external granular and molecular layers. In the internal granular layer a statistically significant, but relatively moderate, rise of 34 per cent occurred. In contrast, in the white matter, a striking 186 per cent increase occurred between birth and day-9.

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Perhaps the most pronounced change in β-glucuronidase occurred between 9 and 14 days in all four layers. In each layer there was a marked and significant decrease to approximately half the activity present at 9 days.

Between 14 days and adulthood the enzymic activity in the molecular layer changed little, whereas in the internal granular layer and white matter the activities decreased significantly. The decrease in white matter was especially pronounced. The activity in this latter layer fell to one-eighth of its level at 14 days.

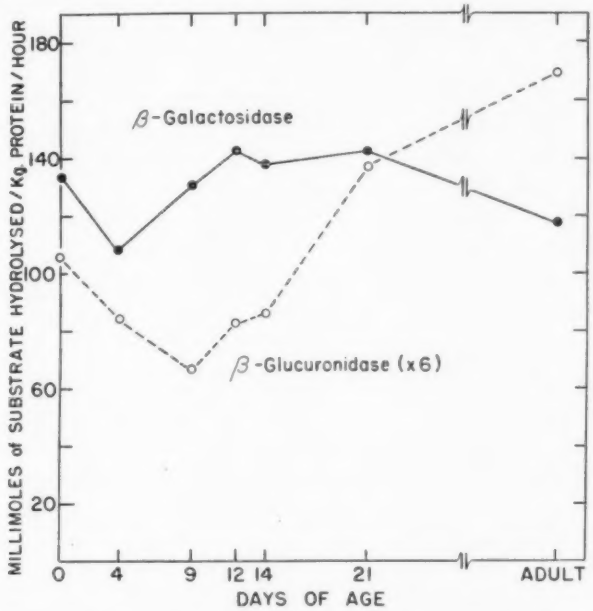

FIG. 1.—Changes in activities of β-galactosidase and β-glucuronidase of whole rat brain during postnatal development. The plotted values are the means from 2(12, 14, 21 day, and adult), 3(4 and 9 day), and 5(0-day) animals at each age for each enzyme. In each instance, values from 2 families were obtained. The agreement between siblings and between families was satisfactory. The minimal and maximal values of individual animals for each enzyme at each age were—β-Galactosidase: 0-day (113, 152); 4-day (101, 118); 9-day (121, 142); 12-day (139, 146); 14-day (127, 149); 21-day (131, 151); adult (104, 130). β-Glucuronidase: 0(16·7, 19·1); 4(13·2, 14·2); 9(9·45, 12·8); 12(13·7, 13·8); 14(14·1, 14·5); 21(22·9, 22·9); adult (27·3, 29·0).

In summary, the changes in the internal granular and white matter layers tended to follow the same pattern but were far more marked in the white matter. The changes in the molecular layer differed quantitatively from those in the internal granular and white matter layers, except for the general decrease in activity in all layers from 9 to 14 days.

β-Galactosidase (Fig. 3) activity in the external granular layer did not show a significant change between 9 and 14 days. It is difficult to evaluate the significance of the decrease from birth to 9 days because of the discrepancy in the enzymic levels of the two newborn animals (Table 2). In the molecular layer there were no significant changes at any time during development, although the decrease from 14 days to adulthood approached the level of significance.

In contrast with these two layers, the internal granular and white matter layers

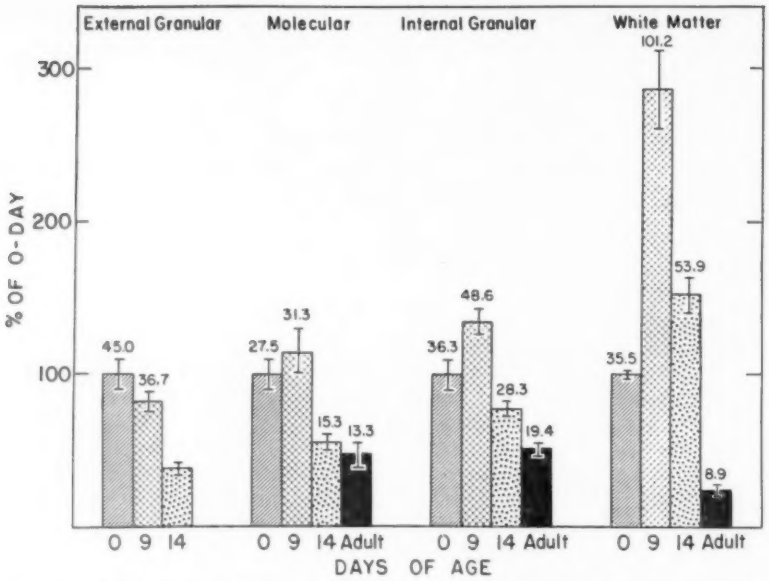

FIG. 2.—β-Glucuronidase per kg lipid-free dry wt per hr in layers of the developing cerebellum. The data for each bar were calculated by dividing the mean activities per kg total dry wt of the two animals (from Table 1) by the corresponding lipid-free dry wt per kg dry wt (calculated from Table 5 in Part I, ROBINS and LOWE, 1961) for each layer at each age. The figures at the top of each bar are activities, expressed as m-moles of substrate hydrolysed per kg lipid-free dry wt per hr. The lines with the cross bar at each end indicate $\pm$S.E.M.

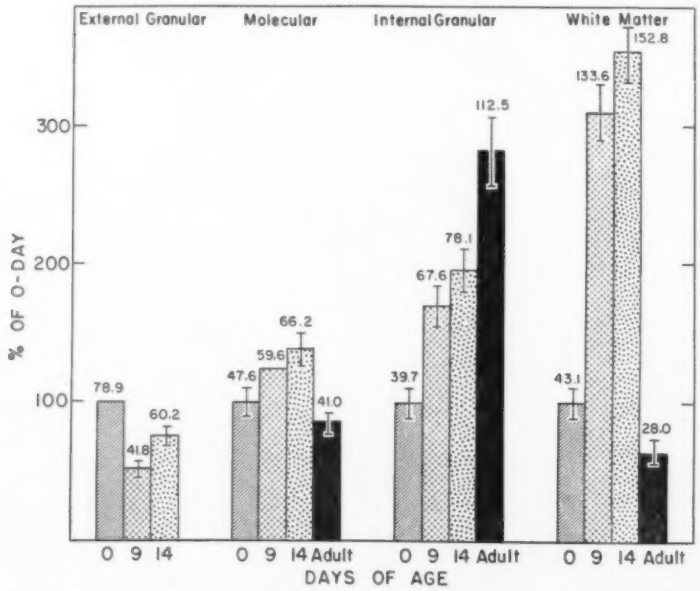

FIG. 3.—β-Galactosidase per kg lipid-free dry wt per hr in layers of the developing rat cerebellum. See legend to Fig. 2 for calculations and meanings of symbols. The activities per kg total dry wt per hr used in the calculations are from Table 2. There are no barred lines indicating S.E.M. in the external granular layer at day-0 and in the molecular layer at day-9, for reasons discussed in the text.

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showed a number of significant changes throughout development. Enzymic levels in the internal granular layer showed a significant increase from birth to 9 days. The increase continued into adult life, although there was only a slight increase from 9 to 14 days.

Changes in white matter were even more marked than in the internal granular layer. A very marked increase in the enzyme in white matter occurred between birth and 9 days, and a further slight increase occurred from day-9 to day-14. In striking contrast to the internal granular layer, however, enzymic activity of the white matter decreased markedly in adulthood to one-sixth its level at 14 days, and below that at birth.

DISCUSSION

In discussing the meaning of these results there are two general limitations regarding their interpretation. Little is known of the biological functions of β-galactosidase and β-glucuronidase, and this lack of knowledge is especially true concerning their possible roles in the function of the nervous system. Secondly, even though histologically homogeneous layers were analysed, with the resulting increase in information concerning histological-chemical correlations, ideally it would be best to analyse the development of individual cell bodies. It then would be possible to make very precise histological-chemical correlations.

The histological differentiation of the molecular layer has been briefly described (ROBINS and LOWE, 1961). There is little change in its appearance until day-8. Between 8 and 14 days there are marked and abrupt histological changes—appearance of Nissl substance, appearance and increase of horizontal cells, differentiation of basket cells, and greatly increased arborizations of the Purkinje cells. By day-14 the molecular layer is well on its way to its adult appearance. Despite these histological changes between 9 and 14 days, β-galactosidase showed a negligible change during this period. β-Glucuronidase, in contrast, decreased by more than half. These findings suggest that neither enzyme is primarily involved in this period of rapid growth. The finding that the activity of β-glucuronidase is greater at 0 and 9 days than at any subsequent time suggests, however, that this enzyme may play a role in preparing the elements of the molecular layer for the period of rapid differentiation, even though its role is minor in the differentiation itself. The role of β-glucuronidase may be to provide free glucuronic acid for the synthesis of a ground substance containing glucuronic acid. (Recent electron microscopic findings which show a paucity of intercellular space in the central nervous system (LUSE, 1960) suggest that this mucopolysaccharide or mucoprotein substance, if it exists, must be largely intracellular). It may be further speculated that in the molecular layer the laying down of a ground substance must precede the period of rapid differentiation. These ideas would then explain the high levels of β-glucuronidase prior to the ninth day and its marked decrease by the fourteenth day.

The differentiation of the internal granular layer shows its most marked changes between 0 and 14 days—rapid increase in numbers of granule cells, grouping into glomeruli of these cells, and great increase in thickness. β-Galactosidase shows a continuing increase in the internal granular layer throughout development, which, however, is least marked from days-9 to -14. The continuing increase of β-galactosidase in the internal granular layer during development contrasts with its behaviour in the molecular layer, where only insignificant changes occur. The increase in the

granular layer also contrasts with the changes in white matter, which show an increase until day-14, followed by a pronounced decrease in adulthood. These differences in behaviour suggest that increased β-galactosidase activity in the adult is associated with cell bodies themselves (the cell-body rich internal granular layer), rather than with their processes (molecular layer) or with white matter. The relationship to cell bodies becomes clearly evident only after 14 days, rather late in the development of the cerebellar cortex.

β-Glucuronidase when compared with β-galactosidase shows a very different pattern of change in the internal granular layer. β-Glucuronidase shows a moderate increase between birth and 9 days, but a marked and continuing decrease beginning at 9 days and continuing into adulthood. The suggestion concerning the significance of these changes is identical with the one already given for its changes in the molecular layer.

The differentiation of white matter during the first 8 days has reached the stage where just visible myelination is occurring. Subsequently myelination reaches its peak rate, from day-9 to day-14 (ROBINS and LOWE, 1961), and continues until about day-40. β-Glucuronidase exhibited an almost 3-fold increase in activity from birth to day-9, following which it decreased markedly and continued to do so into adulthood. This pattern is reminiscent of that in the other layers. The striking increase, however, from birth to 9 days suggests that differentiating glial cells of the presumptive white matter are an especially rich source of the enzyme. These may be active agents in laying down the ground substance referred to above, or at least in providing free glucuronic acid for synthesis. The profound decrease in adulthood indicates that the adult glial cells in white matter have lost the specialized enzymic activity characteristic of the growing glial cells.

β-Galactosidase in the white matter exhibited an almost 4-fold increase by day-14 followed by a marked decrease in adulthood. Between day-9 and day-14 there was a slight increase, in contrast to the marked decrease during this period for β-glucuronidase. This temporal difference between the two enzymes suggests a differing function for them in white matter. The finding that both enzymes decreased in the white matter of the adult suggests, however, that their high values during development are probably related to the presence of growing and differentiating glia.

The decreases in the β-glycosidases in developing white matter, either just before (β-glucuronidase) the onset of the peak rate of myelination or just after (β-galactosidase), contrast with the findings in degenerating central white matter, in which both enzymes showed six- to eight-fold increases (MCCAMAN and ROBINS, 1959). Although it is suggestive to consider these changes as opposite manifestations of a unitary process (synthesis and breakdown of myelin), this interpretation is open to question. It was not possible, in the studies of Wallerian degeneration, to be certain whether the increased enzymic activities were in some element of neural tissue or whether they were carried in by the invading macrophages. If the former alternative could be shown to be true, then it might suggest that proliferating and growing glia (whether during development or in response to injury) are characterized by high levels of these enzymes, whereas adult or 'resting' glia have low levels. Very high levels of β-glucuronidase and β-galactosidase in glial tumours (G. M. LEHRER and H. E. HIRSCH, unpublished) also support the idea that proliferating glia have high levels of these enzymes. LEVVY (1956) has discussed the evidence that high levels of β-glucuronidase

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may be a reflection of a proliferative state of any tissue. He concluded that there was evidence both for and against this hypothesis, and that there may be strain and species differences to explain both kinds of evidence. Work on the nervous system, however, has been limited, and there is the possibility that β-glucuronidase may consistently reflect a state of glial proliferation. The results discussed in the text were obtained on three different species: the rabbit for degenerating central nerve, the rat for the developing cerebellum, and man for the brain tumours.

The changes in the activities of the β-glycosidases in any of the layers studied during development do not show any clear correlation with a presumed 'critical period' occurring between 9 and 14 days postnatally. In the previous study (ROBINS and LOWE, 1961) it was pointed out that of the four enzymes reported there, only lactic dehydrogenase showed an increase beginning during the presumed 'critical period'. Of the six enzymes studied, therefore, five could not be definitely related to the 'critical period'.

β-Glucuronidase has been characterized as one of the hydrolases present in lysosomal particles (lysosomes) in liver (DE DUVE, 1959). Although the identification of lysosomes in brain tissue is not as clear-cut as in liver, preliminary evidence has been presented that they may occur in brain (BEAUFAY, BERLEUR and DOYEN, 1957). If β-glucuronidase is actually representative of lysosomal enzymes in brain, the results of the present investigation indicate that during development these enzymes follow different patterns of development in different histological sites in the nervous system. The results also indicate that in the cerebellum of older animals these hydrolases are present at lower levels than in younger animals. If lysosomal hydrolases have a role in physiological autolysis (DE DUVE, 1959), the high level of β-glucuronidase in the early stages of development suggests that the increased synthesis during growth and differentiation is associated with an increased breakdown of certain cellular constituents. In contrast, in the adult animal, where the levels of β-glucuronidase are lower, there would be a decrease of both synthesis and breakdown of cellular constituents. To ascertain the validity of these interpretations obviously requires studies on other lysosomal hydrolases and on other non-lysosomal enzymes.

SUMMARY

β-Galactosidase and β-glucuronidase activities were measured in the individual layers of the developing cerebellar cortex and in the immediately subjacent white matter of the rat, at birth, in adulthood, and at 9 days and 14 days postnatally.

The patterns of change during development of these two β-glycosidases were different; this result suggests that the enzymes probably have a different function during development.

For each enzyme considered, individually there were striking differences in the patterns of change among the individual layers during development; this result suggests that these histologically distinct layers have a differing β-glycoside metabolism during development. One of the most striking examples of the differences among layers was the continuing increase into adulthood of β-galactosidase in the cell-body rich internal granular layer as contrasted both with the absence of significant change in the synaptic molecular layer and with the pronounced decrease in activity in white matter.

It was suggested that high levels of these enzymes may be characteristic of proliferating glia rather than of 'resting' glia. There is evidence to indicate that glial proliferation in embryological development, Wallerian degeneration, and glial tumours is associated with increased levels of these enzymes.

The high levels of these enzymes in the adult internal granular layer, relative to the other layers, indicate an unknown biological function in nerve cell bodies, presumably unrelated to that in proliferating glia.

Acknowledgement—We wish to thank Miss SHIRLEY MILLSTONE for making the statistical computations.

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THE EFFECT OF IMIPRAMINE ON THE 5-HYDROXYTRYPTAMINE CONTENT AND MONOAMINE OXIDASE ACTIVITY OF THE RAT BRAIN AND ON THE EXCRETION OF 5-HYDROXYINDOLE ACETIC ACID

E. KIVALO, U. K. RINNE and H. KARINKANTA
Department of Physiology, University of Turku, Turku, Finland

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IMIPRAMINE has been reported to be of value in the treatment of depressions in that it alleviates the typical symptoms of motor retardation, fatigue, etc. (KUHN, 1958). It has been shown previously that as a rule antidepressant agents of current clinical interest affect the monoamine oxidase activity (MAO) of the brain and thus the metabolism of 5-hydroxytryptamine (5-HT), noradrenaline and other substances. However, no experimental results concerning the effect of imipramine in this respect can be found in the literature. Only a few personal communications are available (PLETSCHER, 1957; SIGG, 1959; VAN METERS, OWENS and HIMWICH, 1959).

Earlier investigations have shown that imipramine can counteract the tranquillization produced by reserpine and that the reserpine-induced decrease of motor activity in rats can be alleviated by imipramine (VAN METERS, OWENS and HIMWICH, 1959). Furthermore, reserpine is antagonistic to imipramine, reversing the imipramine depression of the reticular formation and causing EEG arousal (VAN METERS, OWENS and HIMWICH, 1959).

In the present investigation the effect of acute as well as chronic imipramine treatment on the 5-HT content and MAO activity of rat brain was studied and the quantity of 5-hydroxyindole acetic acid (5-HIAA) excreted in the urine during chronic treatment was determined. Experiments were made to determine whether imipramine can affect the strong depletion of the 5-HT content of the brain which reserpine is known to produce.

MATERIAL AND METHODS

White male rats weighing 250 g on an average were used. The arrangement of the tests and the dosages of imipramine (Tofranil) and reserpine (Serpasil) can be seen in Tables 1–5. Administration was by intraperitoneal injection. The test and control animals were killed simultaneously by rapid decapitation and their brains (excluding the cerebellum) were immediately excised and weighed.

5-Hydroxytryptamine (5-HT) assay. The brain tissue was ground to a fine consistency in a glass homogenizer in 0·01 N-HCl. The 5-HT was extracted from the homogenate by the acetone–heptane method (KÄRKI and PAASONEN, 1959), and assayed biologically, using the rat stomach method of VANE (1957).

Monoamine oxidase determination. The brain tissue was homogenized with 4 ml distilled water. The monoamine oxidase activity was determined by measuring the disappearance of 5-HT according to BOGDANSKI, WEISSBACH and UDENFRIEND (1957). However 5-HT was determined in these tests by the method described above.

5-Hydroxyindole acetic acid (5-HIAA). This was estimated spectrophotometrically by the method of UDENFRIEND, TITUS and WEISSBACH (1955). One determination was made on the pooled daily urine of 4 to 6 animals.

Statistical treatment. 'Student's' *t*-test (FISHER, 1950) has been used in the statistical treatment of the results.

RESULTS AND CONCLUSIONS

The results of the investigations are given in Tables 1–5.

5-HT content in acute experiments. It can be seen (Table 1) that 4 hr after intraperitoneal injection of imipramine, there was no significant increase in the 5-HT content of the brain until the dosage of impramine was increased to 50 mg per kg body weight. Table 2 shows the 5-HT content of the brain 2 to 26 hr after an imipramine

TABLE 1.—THE EFFECT OF IMIPRAMINE ON THE 5-HT CONTENT OF THE BRAIN

Treatment	Dose (mg/kg body wt)	Number of animals	5-HT (μmg/g wet wt) ±S.E.M.	P*
Physiological saline (control)	—	32	346 ± 10·7	
Imipramine	5	6	345 ± 7·6	>0·05
Imipramine	10	22	361 ± 12·6	>0·05
Imipramine	50	20	405 ± 11·2	<0·001

Test animals killed 4 hr after intraperitoneal injection. Control animals injected with equivalent quantity of physiological saline.

* Compared with the controls.

TABLE 2.—THE 5-HT CONTENT OF THE BRAIN 2 TO 26 HOURS AFTER IMIPRAMINE INJECTION

Treatment (50 mg/kg body wt.)	Time after injection (hr)	Number of animals	5-HT (μmg/g wet wt) ±S.E.M.	P*
Control	—	7	337 ± 9·4	
Imipramine	2	5	400 ± 12·4	<0·01
Imipramine	4	6	410 ± 28·0	<0·05
Imipramine	9	6	433 ± 21·3	<0·01
Imipramine	18	5	406 ± 20·7	<0·01
Imipramine	26	6	395 ± 25·6	<0·05

* Compared with the controls.

TABLE 3.—THE EFFECT OF IMIPRAMINE ON THE 5-HT DEPLETION CAUSED BY RESERPINE

Treatment	Number of animals	5-HT (μmg/g wet wt.) ±S.E.M.	P*	P†
Reserpine (5 mg/kg)	10	114 ± 6·4		<0·001
Imipramine (10 mg/kg) + Reserpine (5 mg/kg)	5	96·8 ± 8·3	>0·05	<0·001
Imipramine (50 mg/kg) + Reserpine (5 mg/kg)	6	97 ± 25·4	>0·05	<0·001

Reserpine injected 1 hr after imipramine injection. Animals killed 5 hr after reserpine injection.

* Compared with the reserpine group.

† Compared with the controls.

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TABLE 4.—THE EFFECT OF TEN DAYS' IMIPRAMINE TREATMENT ON THE 5-HT CONTENT OF THE BRAIN

	Daily dosage	Number of animals	5-HT (μmg/g wet wt) ±S.E.M.	P*
Controls	—	4	375 ± 13·6	
Controls	saline	11	397 ± 9·8	>0·05
Imipramine	10 mg/kg	6	452 ± 12·0	<0·01
Imipramine	50 mg/kg	4	458 ± 2·8	<0·001

Animals killed 4 hr after the last injection.
* Compared with the controls.

TABLE 5.—THE EFFECT OF DAILY INJECTION (50 MG/G BODY WT) OF IMIPRAMINE ON 5-HIAA EXCRETION

Duration (days)	5-HIAA in μmg per 100 g body wt per 24 hr*		
	Controls	Saline	Imipramine
2	19·6 (6)		11·9 (6)
3	6·5 (6)	13·5 (6)	15·0 (6)
4	13·4 (6)	29·5 (6)	15·3 (5)
5	10·2 (6)	20·2 (6)	15·4 (4)
6		15·4 (6)	17·5 (4)
7	13·9 (6)	16·0 (6)	10·5 (4)
8	13·6 (6)	11·0 (6)	29·6 (4)
9	12·6 (6)	16·6 (6)	
10	13·9 (6)	11·1 (6)	
Mean ± S.D.	12·96 ± 1·3	16·66 ± 2·12	16·46 ± 2·36

* Mean for 4–6 animals (No. in brackets).

injection of 50 mg per kg body weight. It can be seen that a significant increase in 5-HT occurs within 2 hr and that the effect is at its maximum 9 hr after imipramine injection. In the acute test (Table 3) reserpine caused a highly significant depletion of the 5-HT content in the brain ($P < 0·001$). This depletion could not be prevented by injection of 10 mg or by 50 mg imipramine per kg body weight, given one hour before reserpine.

5-HT content in chronic experiments. It can be seen (Table 4) that a significant increase in the 5-HT content of the brain occurs after ten days' imipramine treatment with either 10 mg or 50 mg per kg body weight.

Monoamine oxidase activity. In animals which were injected with 10 mg imipramine per kg body weight and killed 4 hr later the MAO activity was 4·1 per cent higher than in the controls; in animals receiving 50 mg per kg it was 0·9 per cent lower than in the controls but there were no statistically significant differences. It can be said therefore that inhibition of monoamine oxidase by imipramine was not observed.

5-HIAA. Data on the excretion of 5-HIAA during the ten days when daily injections of 50 mg imipramine per kg body weight were given (Table 5) show that there was no

significant increase in comparison with the untreated controls or with the saline-treated animals.

The investigation revealed that imipramine is not a monoamine oxidase inhibitor but nevertheless causes an increase in the 5-HT concentration of the brain. It is not known what kind of mechanism is responsible, but imipramine was unable to prevent the 5-HT depletion in the brain caused by reserpine. It seems that the brain level of 5-HT increases when the dose of imipramine is increased to 50 mg per body weight; this is shown more clearly in chronic tests than in acute tests although an increase in the 5-HT content was observed within two hours after imipramine administration. The fact that there was no significant increase in the amount of 5-HIAA excreted in comparison with the controls, despite the increase in the 5-HT content of the brain after imipramine administration, is obviously attributable to the relatively low increase of 5-HT concentration so that the consequent variations in excretion of 5-HIAA are not prominent enough.

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SUMMARY

The effect of imipramine on the 5-hydroxytryptamine (5-HT) content and monoamine oxidase activity of the rat's brain and on the urinary excretion of 5-hydroxyindole acetic acid (5-HIAA) was studied. Imipramine in a single dosage of 50 mg/kg body weight caused a highly significant increase in the 5-HT concentration of the rat's brain. In the prolonged tests there was a significant difference from the controls at a daily dosage of 10 mg/kg body weight daily. Imipramine was unable to prevent the depletion of 5-HT in the brain caused by reserpine. It had no significant effect on the monoamine oxidase activity of the brain. Imipramine caused no change in the urinary excretion of 5-HIAA in the long term test with a daily dosage of 50 mg/kg body weight.

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THE ACTION OF SOME α-METHYL AND OTHER AMINO ACIDS ON CEREBRAL CATECHOLAMINES*

T. L. SOURKES, G. F. MURPHY, BEATRIZ CHAVEZ and MARIE ZIELINSKA
Allan Memorial Institute of Psychiatry and McGill University,
Montreal, Canada

(*Received* 24 *April* 1961)

IT HAS been shown recently that AMDP†, at first studied as an inhibitor of mammalian DOPA† decarboxylase *in vitro* and of the tyrosine decarboxylase of *Str. faecalis* R (SOURKES, 1954), acts *in vivo* upon the decarboxylation of DOPA (DENGLER and REICHEL, 1958; MURPHY and SOURKES, 1959), 5-hydroxytryptophan (WESTERMANN, BALZER and KNELL, 1958; SMITH, 1960; OATES, GILLESPIE, UDENFRIEND and SJOERDSMA, 1960), tyrosine and tryptophan (OATES *et al.*, 1960). This action on the decarboxylation of DOPA *in vivo* is also displayed by α-methyl-3-hydroxyphenylalanine (α-methyl-*m*-tyrosine) (SOURKES, MURPHY and WOODFORD, 1960) as well as by α-methyl-2-hydroxyphenylalanine (α-methyl-*o*-tyrosine), α-methyl-2:5-DOPA, and α-methyl-5-hydroxytryptophan (MURPHY and SOURKES, 1961). By use of fluorometric methods it has been shown (MURPHY and SOURKES, 1959) that AMDP administered parenterally to the rat has two effects upon the cerebral catecholamines: one of decreasing the concentration of the endogenous amines and, secondly, of interfering with the conversion of exogenous DOPA to cerebral dopamine and noradrenaline. Although the injected AMDP is found in the brain in concentrations known to be effective for the inhibition of DOPA decarboxylase the rapid decrease in cerebral catecholamines could conceivably be due to some other mechanism than through inhibition of the decarboxylation. In this paper the amine-depleting action of AMDP has been further studied. Moreover, some other amino acids have been tested to compare their ability with that of AMDP in depleting the brain of its catecholamines.

EXPERIMENTAL

Male rats of the Sprague-Dawley strain were purchased locally. When they reached 95–120 g in body weight they were assigned to treatment by a procedure using random numbers, and were injected intraperitoneally with AMDP or other amino acid dissolved in saline. The volume of solution injected was uniformly 1 ml per 100 g body weight. Because of their low solubility, tryptophan and α-methyl-5-hydroxytryptophan were ground with the requisite volume of saline in a Potter-Elvehjem homogenizer and were injected as suspensions. The rats were guillotined at the indicated times following the injection. The brains were quickly removed, rinsed free of visible blood, blotted on filter paper, weighed, and minced with scissors in 10% trichloroacetic acid. At a convenient time within the next hour the mincing was completed in a Potter-Elvehjem homogenizer. Purification of the catecholamines and of AMDP was carried out on alumina and the acetic acid eluates thereby obtained were examined fluorometrically for dopamine, noradrenaline and AMDP by methods described previously (DRUJAN, SOURKES, LAYNE and MURPHY, 1959; MURPHY and SOURKES, 1959; SOURKES and MURPHY, 1961).

* A preliminary report of some of the data in this paper was given at the International Symposium on '*The Extrapyramidal System and Neuroleptics*', Université de Montréal, November 17–19, 1960 (SOURKES, 1961).

† The abbreviations used are: AMDP, α-methyl-3:4-dihydroxyphenylalanine; DOPA, 3:4-dihydroxyphenylalanine.

5-Hydroxyindoles were measured in various organs after the injection of α-methyl-5-hydroxytryptophan by reacting portions of neutral, protein-free extracts of the organs with nitrosonaphthol, and measuring the optical density of the coloured complex (UDENFRIEND, WEISSBACH and BRODIE, 1958). When other amino acids were used, only dopamine and noradrenaline were estimated.

The following amino acids were generously provided by Merck Sharp and Dohme Research Laboratories, Rahway, New Jersey (Lot number in parentheses): AMDP (L516530–05); α-methyl-*m*-tyrosine monohydrate (L523766–1–3); α-methyl-*o*-tyrosine monohydrate (L585449–0–2); α-methyltryptophan (54R2726); α-methyl-5-hydroxytryptophan (L564448–0–7); and α-methylphenylalanine (L511525–0–5). These compounds were racemic mixtures. The other amino acids used were purchased commercially.

RESULTS

Effect of AMDP on cerebral catecholamines

AMDP in amounts ranging from 52·75 to 422 mg (0·25 to 2 m-moles) per kg body weight was injected into groups of 6 rats. Three hours later the animals were killed and the cerebral dopamine and noradrenaline were measured (Fig. 1). The concentration

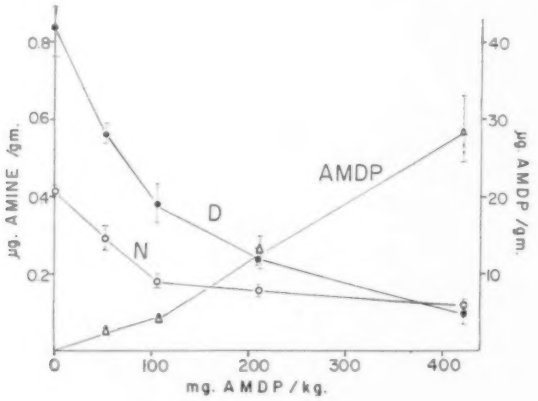

FIG. 1.—Effect of varying dosages of AMDP upon the concentration of cerebral catecholamines. DL-AMDP injected intraperitoneally. Dopamine or noradrenaline (μg/g wet brain). The rats were killed 3 hr after the injection of the amino acid, and the brains were removed for analysis immediately. The plotted points are mean values; the vertical lines through them represent S.E.M.

of dopamine showed a proportionately greater decrease than the noradrenaline concentration for equivalent doses of AMDP, but both amines were reduced to 50 per cent of their normal concentration, in the 3-hr period studied, by a dose of approximately 100 mg DL-AMDP/kg. The mean concentration of AMDP in the brain 3 hr after injection of this dose was 4·3 μg/g (0·02 m-mole/kg).

To determine the rate at which the concentration of the cerebral catecholamines declines, rats were injected with 1·0 m-mole of AMDP/kg, and groups of two to six rats were killed at intervals thereafter. The results of several such experiments have been pooled (Table 1). AMDP rapidly entered the brain, but its concentration also declined rapidly so that it was not always detectable 6 hr after the injection, and never at 24 hr. Dopamine fell sharply in concentration after the injection. Noradrenaline appeared to increase slightly (0·5–1 hr), but thereafter fell in concentration somewhat more slowly than the dopamine concentration. Within 6 hr the dopamine level had

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returned to the normal range, but the noradrenaline concentration remained depressed for at least 72 hr.

Effect of other amino acids

The effects of α-methyl-*m*-tyrosine and of α-methyl-5-hydroxytryptophan were measured in the same way as for AMDP, to determine how general the amine-depleting

TABLE 1.—EFFECT OF AMDP ON THE CONCENTRATION OF CEREBRAL CATECHOLAMINES

Duration of action* (hr)	No. of rats	AMDP†	Dopamine	Noradrenaline
		(Mean ± S.E.M. in μg/g)		
(Controls)	20	—	0·57 ± 0·017	0·39 ± 0·013
0·5–1	4	21·47 ± 4·494	0·01 ± 0·065	0·46 ± 0·026
1·5–2	4	24·40 ± 6·078	0·02 ± 0·103	0·30 ± 0·020
2·5–3	6	12·24 ± 2·313	0·07 ± 0·09	0·24 ± 0·038
6	2	0·80, 0·00	0·46, 0·62	0·09, 0·27
24	8	0	0·60 ± 0·035	0·18 ± 0·011
48	4	0	0·52 ± 0·067	0·20 ± 0·023
72	3	0	0·54 ± 0·044	0·26 ± 0·015

* Time after the injection of AMDP at which the respective groups of rats were killed.

† DL-AMDP was injected intraperitoneally at a rate of 1·0 m-mole per kg body weight.

action is for α-methyl amino acids. Both amino acids were injected intraperitoneally in a dose of 1·0 m-mole/kg body weight. The results (Figs. 2 and 3), show that α-methyl-*m*-tyrosine, like AMDP, causes a loss of cerebral catecholamines, though the loss does not occur quite as rapidly. Moreover, in the case of rats given α-methyl-*m*-tyrosine the cerebral dopamine requires more than 24 hr to attain the normal mean level. α-Methyl-5-hydroxytryptophan shows a somewhat different pattern producing a very slight decrease in the cerebral dopamine and a more considerable one in the case of the noradrenaline. This compound was injected as a fine suspension, which may have

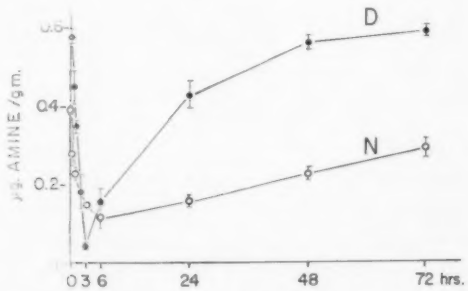

FIG. 2.—Effect of DL-α-methyl-*m*-tyrosine upon the concentration of cerebral catecholamines. The amino acid (1·0 m-mole/kg body wet) was injected intraperitoneally at 'zero' time, and groups of rats were killed at various times thereafter.

limited its rate of absorption. Indeed, attempts to detect excess amounts of 5-hydroxyindoles in the brain by the nitrosonaphthol method yielded negative results, although small amounts were detected in the liver and larger amounts in the kidneys at 1–3 hr. It is also possible that α-methyl-5-hydroxytryptophan has an intrinsically weak action upon the cerebral catecholamines, but additional experiments are necessary to establish this point. In experiments with α-methyl-*o*-tyrosine and α-methylphenylalanine (1·0 m-mole/kg) there was no effect upon the cerebral catecholamines as measured by their concentrations 3 hr after the injection of the compound.

Other amino acids were also tested (Table 2). It appears that all amino acids which were allowed to act for the 45-min interval, had some effect upon the cerebral dopamine and that restoration of the normal levels of this amine was under way in 3 hr. The depletory effect was greater on the dopamine than on the noradrenaline level. DL-Methionine appeared to have no depletory effect, but it was tested at 3 hr only.

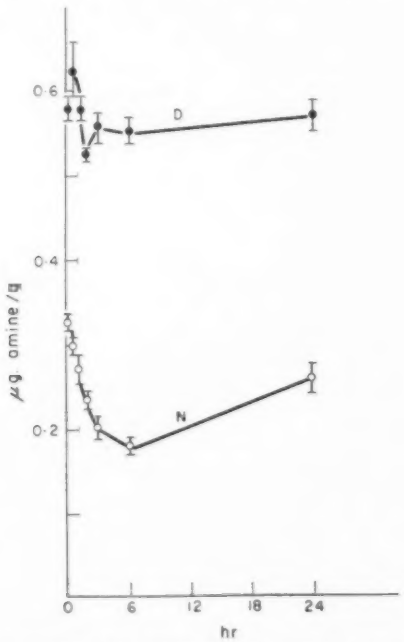

FIG. 3.—Effect of DL-α-methyl-5-hydroxytryptophan upon the concentration of cerebral catecholamines. The amino acid (1·0 m-mole/kg body wt) was injected intraperitoneally as a suspension in saline at 'zero' time and groups of rats were killed at various times thereafter.

DISCUSSION

The action of AMDP in reducing the cerebral amines, as described by MURPHY and SOURKES (1959) and SMITH (1960), has now been shown to occur in two stages. The first is characterized by the loss of dopamine and noradrenaline from the brain, and resembles the depletion which follows the administration of reserpine (*cf.* SMITH, 1960); the peak of this effect is reached in 3–6 hr. The second stage is one of repletion, and it differs for the two amines.* It can be seen (Table 1) that once the concentration

* It has been reported that 5-hydroxytryptamine of guinea pig brain is also released through the action of AMDP (HESS, CONNAMACHER and UDENFRIEND, 1961), and that this amine is resynthesized within 24 hr.

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of AMDP in the brain has fallen to a low level the resynthesis of dopamine occurs at an average rate of 0·1 μg/g of brain per hr, or greater. If this rate were maintained for an extended period the observed concentration of dopamine in the brains of control rats could be turned over in 5–6 hr. The very slow rate of repletion of noradrenaline contrasts with this rapid turnover of dopamine. It can be inferred that the resynthesis of dopamine commences as soon as the concentration of AMDP in the

TABLE 2.—EFFECT OF SOME AMINO ACIDS UPON THE CONCENTRATION OF CEREBRAL CATECHOLAMINES

Amino Acid	Period after injection (min)	Dopamine	Noradrenaline
		(μg of amine/g of brain)*	
Control	45	0·74, 0·70, 0·81	0·68, 0·68, 0·71
DL-*m*-Tyrosine		0·34, 0·58, 0·34	0·38, 0·63, 0·27
DL-*o*-Tyrosine		0·63, 0·55, 0·52	0·53, 0·53, 0·51
DL-5-Hydroxytryptophan		0·53, 0·46, 0·57	0·56, 0·49, 0·50
L-Tryptophan		0·42, 0·53, 0·63	0·56, 0·50, 0·47
DL-Alanine		0·41, 0·51, 0·42	0·39, 0·62, 0·57
Control	90	0·95, 0·76, 0·86, 0·69	0·40, 0·46, 0·41, 0·41
DL-*m*-Tyrosine		0·23, 0·25	0·44, 0·22
DL-*o*-Tyrosine		0·25, 0·76	0·29, 0·34
DL-5-Hydroxytryptophan		0·66, 0·62	0·37, 0·30
DL-*m*-Tyrosine	180	0·47, 0·20	0·26, 0·47
DL-*o*-Tyrosine		0·63, 0·79	0·36, 0·38
DL-5-Hydroxytryptophan		0·87, 0·34	
DL-Methionine		0·79, 0·82	0·48, 0·44

* Individual values are given.

brain decreases to a level which is ineffective for enzymic inhibition. This further implies that the immediate amino acid precursor of the catecholamines is also rapidly synthesized, for there is no evidence that the brain contains large stores of a precursor such as DOPA which could be drawn upon quickly for conversion to dopamine. Oxidation of dopamine to noradrenaline is known to be a slow step in biosynthesis, and this fact could account for the delay in the return of noradrenaline to normal levels in the brain.

As a catechol amino acid, AMDP forms a fluorophore through oxidation and can therefore be expected to interfere in the determination of the endogenous catecholamines. This is a serious problem when the catecholamines are to be determined in, say, the urine after administration of large amounts of AMDP. The difficulties have been averted in the present studies by choice of methods ('Procedure D', MURPHY and SOURKES, 1959); noradrenaline can then be measured in the brain extracts under conditions where the oxidation product of AMDP does not fluoresce, and dopamine can be determined under related conditions where an equivalent amount of AMDP yields but 1–5 per cent as much fluorescence. For more general use as an anti-decarboxylase *in vivo*, a non-catecholic compound would be preferable. α-Methyl-*m*-tyrosine offers this advantage, for it does not interfere with the determination of the catecholamines under any conditions thus far tested in this laboratory; it inhibits

decarboxylation of DOPA *in vivo* (MURPHY and SOURKES, 1961); and it acts like AMDP upon the cerebral catecholamines (Fig. 2). α-Methyl-5-hydroxytryptophan, although effective against DOPA decarboxylase *in vivo* (MURPHY and SOURKES, 1961), did not prove to have a potent depleting action upon the brain amines. The fact that the loss of cerebral catecholamines can be provoked by several 'normal' amino acids, *i.e.* by amino acids possessing the α-hydrogen atom, indicates that this depletion is a general type of action. Indeed, there is the possibility that the amino acids act, at least in part, after decarboxylation to their amines. Recently AXELROD and TOMCHICK (1960) have demonstrated that the administration of certain sympathomimetic amines, followed by the injection of isotopically labelled adrenaline and noradrenaline, results in a more rapid metabolism of the exogenous catecholamines. They have suggested that the noncatecholic amines compete for storage sites which would be normally available to the catecholamines, so that the latter compounds are more readily degraded metabolically. A similar mechanism may be applicable to the endogenous catecholamines vis-à-vis a relatively large amount of some 'foreign' amine derived by decarboxylation of an injected amino acid like AMDP, *m*-tyrosine, and others mentioned in this paper. That is, amines formed *in situ* from the injected amino acid may displace the already bound native amines and cause their loss from the tissue. It must also be kept in mind that the decarboxylation of α-methyl amino acids gives rise to amines bearing the isopropylamine side-chain and that such amines, the prototype of which is amphetamine, are inhibitors of monoamine oxidase. It is therefore conceivable that AMDP, entering the cerebral cell, is converted in part to 3:4-dihydroxyamphetamine ('α-methyldopamine') which might then exert anti-monoamine oxidase activity; such a metabolic process would militate against diminution of the cerebral amines. From the data reported here this did not play an important role. In the case of rats treated with AMDP, no evidence of dihydroxyamphetamine was found in the brain, although chronic treatment of adult humans with 1–1·5 g of AMDP daily by mouth leads to the appearance of its decarboxylation product in the urine in small amounts (SOURKES, MURPHY and CHAVEZ-LARA, 1961).

The differential rates at which dopamine and noradrenaline are lost from the brain and later resynthesized signify the possibility of eventually finding agents which will specifically deplete one or the other of these two catecholamines. This should prove highly useful in elucidating their respective cerebral functions.

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SUMMARY

Two inhibitors of aromatic amino acid decarboxylase, α-methyl-3:4-dihydroxyphenylalanine (AMDP) and α-methyl-*m*-tyrosine, cause a rapid depletion of cerebral catecholamines, upon intraperitoneal injection into the rat. The loss of noradrenaline is long-lasting, but the dopamine content of the brain is restored quickly, at a rate as high as 0·1 μg/g of brain/hr. The action of AMDP upon the dopamine level has been shown to persist as long as it is present in the brain in concentrations known to be effective for inhibition of DOPA decarboxylase. Some 'normal' amino acids, *i.e.* those possessing an α-hydrogen atom, can also cause a loss of cerebral catecholamines.

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FREE NUCLEOTIDES OF THE BRAIN IN VARIOUS MAMMALS

PAUL MANDEL and SUZANNE HARTH
Institut de Chimie Biologique, Faculté de Médecine, Strasbourg, France

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INTEREST in the study of the free nucleotides of the tissues results from the fact that adenine nucleotides play an essential part in energy metabolism and in transphosphorylation (LIPMANN, 1941; KORNBERG, 1951). Cytosine nucleotides participate in the biosynthesis of lipids (KENNEDY and WEISS, 1956), and the nucleotides of uracil and guanine take part in the synthesis of polysaccharides (LELOIR, 1957); guanosine triphosphate appears to be concerned in the biosynthesis of proteins.

Many authors have studied the easily hydrolysable phosphate esters of the acid-soluble fraction of the brain, either directly or by the method using the barium and calcium salts (KERR, 1935, 1941; STONE, 1943; DAWSON and RICHTER, 1950; ALBAUM, TEPPERMAN and BODANSKY, 1946). KRATZING and NARAYANASWAMI (1953) as well as THORN, PFLEIDERER, FROWEIN and ROSS (1955) have carried out enzymic estimations. However, these methods gave no information about the content of the nucleoside polyphosphates, ADP, GTP, UTP, UDP, CTP and CDP.* Recently, by means of column chromatography, the distribution of free nucleotides was determined in rat brain (SCHMITZ *et al.*, 1954*a*, *b*) and in mouse, rat and rabbit brain (MANDEL and HARTH, 1957; MANDEL, HARTH and REBEL, 1958). DOHRING and GERLACH (1957) used paper chromatography to estimate several of the free nucleotides in the brain and HEALD (1956) used electrophoresis for the same purpose.

It has been maintained that excitation of the nervous system during decapitation could lead to a reduction in energy-rich compounds (STONE, 1940; KRATZING and NARAYANASWAMI, 1953; DOHRING and GERLACH, 1957; WEIL-MALHERBE, 1953). COPER, KERKEN and KORANSKY (1954) reported satisfactory values for animals decapitated without anaesthesia. DOHRING and GERLACH (1957) obtained high values for ATP in animals killed under anaesthesia but found no appreciable difference in the values for GTP, UTP, ADP and AMP. One objection to killing animals under anaesthesia has been that ATP may accumulate because of lack of utilization (MCILWAIN, BUCHEL and CHESHIRE, 1951; STONE, 1940).

Consideration of these facts prompted us to study the distribution of the nucleotides of the brain in various species of mammals, in order to determine the best way of obtaining brain material which would give reliable values.

EXPERIMENTAL

Preliminary tests showed that chloral hydrate appeared to have the least effect on the concentrations of nucleotides. In the first experiments normal mice or mice under narcosis induced by chloral hydrate

* *Abbreviations:* AMP, ADP, ATP, 5-adenosine mono-, di- and triphosphate; GMP, GDP, GTP, 5-guanosine mono-, di- and triphosphate; CMP, 5-cytidine monophosphate; CDP-chol., 5-cytidine diphosphate choline; CTP, 5-cytidine triphosphate; UMP, UDP, UTP, 5-uridine mono-, di- and triphosphate; UDPG, 5-uridine di-phosphate glucose; UDPA, 5-uridine diphosphate *N*-acetylglucosamine; UDPCo, coenzyme of UDP taken as a whole; IMP, 5-inosine monophosphate; DPN, diphosphopyridine nucleotide.

(30 mg per 100 g body weight) for 1 min were plunged whole into liquid nitrogen. The frozen animals were decapitated and the brains were isolated in a manipulation cabinet cooled below 0° (MANDEL, 1959).

In the second series rats were used in 4 groups: (1) normal rats and (2) rats anaesthetized for 3 min, were decapitated rapidly, the heads were frozen in liquid nitrogen and the brains dissected out below 0°; (3) normal rats were decapitated, the fresh brain rapidly dissected out and then frozen; and (4) whole rats were frozen, then decapitated and the brains removed. In other experiments normal and anaesthetized rabbits were decapitated, the heads frozen in liquid nitrogen and the brains removed. For guinea pigs, only decapitation under chloral narcosis was used.

Decapitation of anaesthetized animals was carried out at the moment when the animal no longer reacted to a bodily displacement and the conjunctival, but not the corneal, reflex had disappeared.

The experiments were carried out with 220 mice (18–25 g), 140 rats (120–180 g), 25 guinea pigs (250–350 g) and 23 rabbits (2–2·5 kg).

The cerebral hemispheres were homogenized in a mechanical Potter grinder with 4 vols. of ice-cold aqueous ethanol (50 per cent v/v.). The homogenate was centrifuged at 20,000 rev/min (80,000 g) for 30 min in a refrigerated Spinco L centrifuge. The residue was extracted a second time with 2 vols. of ice-cold aqueous ethanol and centrifuged in the same conditions. The two supernatants were mixed and acidified to 0·3 N with 0·6 N-perchloric acid. The precipitate was removed by centrifugation. The acid-soluble fraction was then neutralized to pH 7 with aqueous potassium hydroxide. The solution was left to stand for 1 hr at 0° to complete precipitation of potassium perchlorate. After a further centrifugation, the supernatant liquid was frozen and lyophilized. The dry residue was taken up in a few ml of twice distilled water and the solution was analysed by column chromatography (Dowex 1 × 8) according to the method of COHN (1950) modified by SCHMITZ *et al.* (1954*a*, *b*). The fractions isolated were lyophilized to eliminate formic acid and formate and then subjected again to column or paper chromatography in order to obtain pure compounds (MANDEL, HARTH and REBEL, 1958). Quantitative determinations were made by measuring absorption in the ultra-violet range at wavelengths of 2600 and 2750 Å and by estimations of phosphorus (BRIGGS, 1922), and, in the case of purine nucleotides, of ribose (MEJBAUM, 1939). The molecular proportions of phosphorus and ribose and the absorption in the ultra-violet range determined the degree of phosphorylation of the various nucleotides. The different methods of chromatography permitted the identification of the base of each nucleotide.

RESULTS

Results of chromatographic analysis of the brains of normal mice frozen whole (Fig. 1) and of rapidly decapitated normal rats (Fig. 2) showed high peaks for ATP, GTP and UTP, the most highly phosphorylated nucleotides, but only insignificant amounts of AMP and GMP were found.

Comparison of the results obtained for anaesthetized and unanaesthetized mice plunged whole into liquid nitrogen (Fig. 3) showed that the differences were small and not significant. Slightly less ATP and slightly more UTP were found in anaesthetized mice.

A comparison of results for unanaesthetized rats (Table 1, column 3) and rats anaesthetized for periods up to 3 min (Table 1, column 4) shows that the differences for nucleoside triphosphates were small and not significant. In both groups only traces of GMP and UMP were found. The value for AMP was slightly greater after anaesthesia but still low (2·5 μmole/100 g wet weight). When the rats were frozen whole (Table 1, column 1), the values for ATP were only 12 per cent of those obtained for rats decapitated before freezing; values for ADP were 1·5–4·4 times greater, and those for AMP were 35–45 times higher. Values for guanine and uracil nucleotides showed similar changes; GTP decreased by more than half, GDP was 1–6 times higher and GMP increased from only traces to 21·3 μmoles per 100 g wet weight. The values for IMP were 2–5 times higher for rats frozen whole.

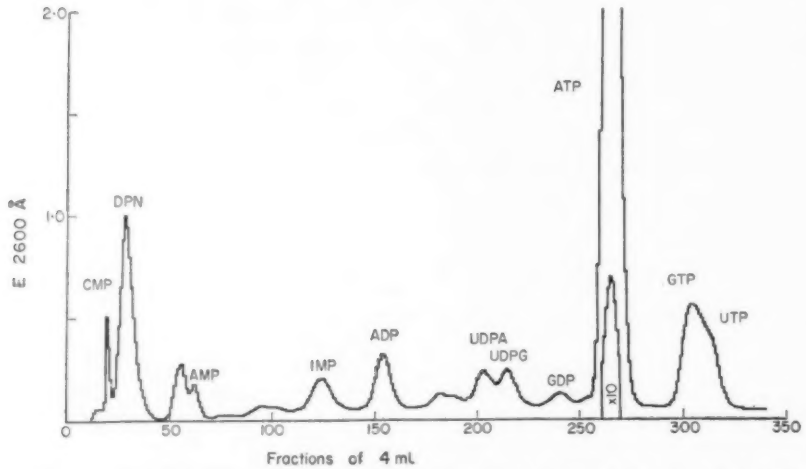

FIG. 1.—Chromatography of the free nucleotides of the cerebral hemispheres of mice. The pooled cerebral tissue weighed 9 g and was obtained from 30 mice. Column: Dowex 1 × 8; formate 0·8 × 20 cm; Mixer: 350 ml; Reservoir 0·4 N-formic acid at the beginning, changed to 4·0 N-formic acid at tube 45, to 4·0 N-formic acid + 0·2 M-ammonium formate at tube 140, to 4·0 N-formic acid + 0·8 M-ammonium formate at tube 230. Rate: 2 ml per min. Fractions of 4 ml.

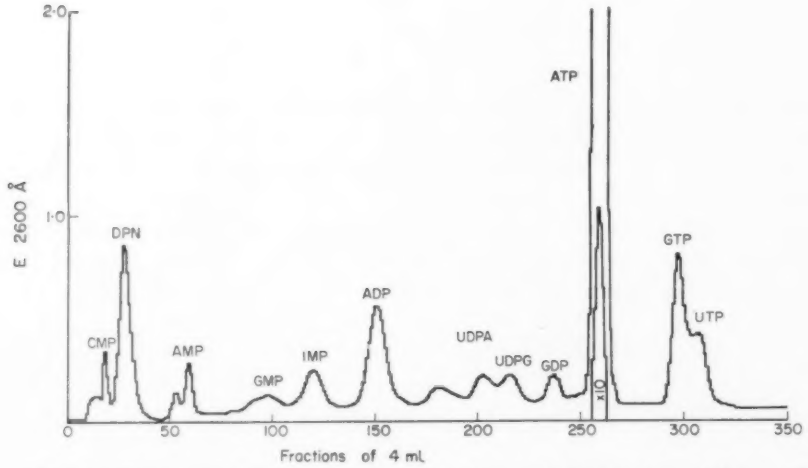

FIG. 2.—Chromatography of the free nucleotides of the cerebral hemispheres of the rat. The pooled cerebral tissue weighed 8·9 g and was obtained from 12 rats. Column operation as in Fig. 1.

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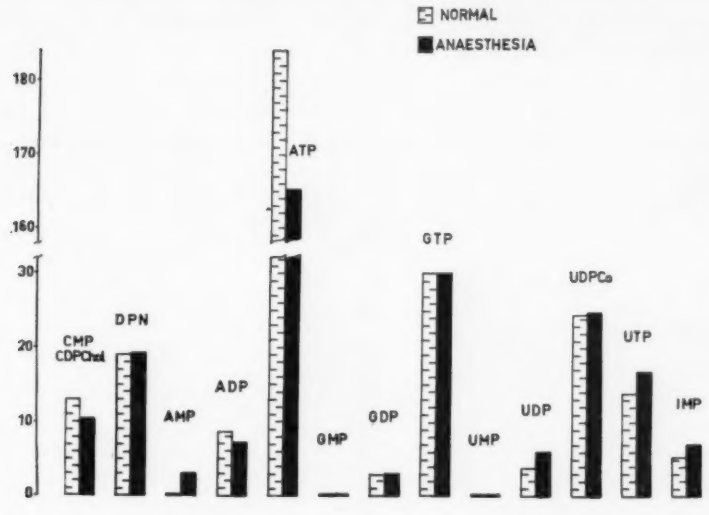

FIG. 3.—Free nucleotides of the cerebral hemispheres of normal and anaesthetized mice. Values in μmoles per 100 g wet weight. Mean of 30 brains of anaesthetized mice; 60 brains of normal mice. GMP and UMP were present as traces in normal and anaesthetized mice; AMP as a trace in normal mice and a little more in anaesthetized mice.

TABLE 1.—COMPARISON OF THE FREE NUCLEOTIDES OF RAT BRAIN FOR DIFFERENT METHODS OF SACRIFICE

(μmoles per 100 g wet weight)

Nucleotides	1	2	3	4
CMP	12·3	24·0	14·5	12·0
DPN	7·3	2·0	12·2	25·0
AMP	116·3	104·0	2·5	3·2
ADP	45·5	62·0	18·2	8·5
ATP	17·1	37·7	140·5	143·0
GMP	21·3	21·9	traces	traces
GDP	25·3	17·5	10·6	3·7
GTP	10·0	12·5	24·5	27·0
UMP	15·8	7·8	traces	traces
UDP	7·1	4·3	3·5	2·1
UDPCo	18·0	22·9	21·3	27·0
UTP	4·3	3·9	12·5	15·9
IMP	39·1	25·3	12·7	6·6

1. Rats plunged whole in liquid nitrogen (28 rats).
2. Rats decapitated, brain dissected (20 sec) then frozen in liquid nitrogen (72 rats).
3. Rats decapitated, heads frozen in liquid nitrogen (24 rats).
4. Rats anaesthetized, decapitated, heads frozen in liquid nitrogen (9 rats).

Comparison of values obtained when fresh brain was dissected whole and then frozen (Table 1, column 2) with the values for anaesthetized rats decapitated before the heads were frozen (Table 1, column 4) showed that the ATP, GTP and UTP values for fresh brain were 26, 46, and 25 per cent of those for anaesthetized rat brain, the ADP, GDP and UDP values for fresh brain were, respectively, 6·3, 3·6 and 1·0 times higher, and the AMP and IMP values were 31·5 and 2·8 times higher, respectively. The GMP and UMP contents increased from trace level in anaesthetized rats to 21·9 and 7·8 μmoles respectively per 100 g wet weight in fresh brain. The UDPCo value remained within the range of physiological variation.

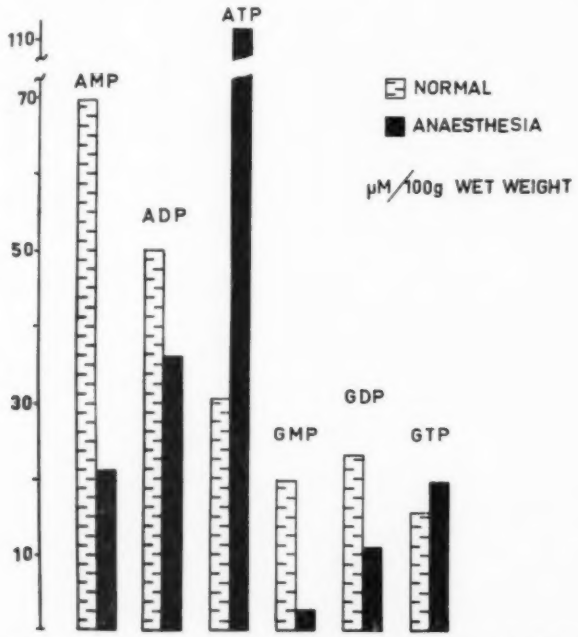

FIG. 4.—Free nucleotides of the cerebral hemispheres of normal and anaesthetized rabbits. Values in μmoles per 100 g wet weight. Average of 6 brains of normal rabbits; 6 brains of anaesthetized rabbits.

Comparison of the adenylic and guanylic nucleotide levels for rabbits (Fig. 4) showed that for anaesthetized animals the ATP and GTP values were 2·6 and 0·2 times greater than for unanaesthetized rabbits, whereas the AMP and GMP values were about 70 and 90 per cent less.

Comparison of the contents of free nucleotides in the cerebral hemispheres of mice, rats, guinea pigs and rabbits showed that, in all species, ATP was by far the most abundant nucleotide. We do not think that the relatively low values for rabbits and guinea pigs represent the situation *in vivo*. The values for GTP were fairly close for mice and rats, 30·0 and 25·4 μmoles per 100 g fresh weight, respectively, and were lower for rabbits and guinea pigs, 19·4 and 16·3 μmoles per 100 g fresh weight. The values for UTP showed similar differences. Very little IMP was found in mouse and rat brain, but 2–4 times as much (about 20 μmoles/100 g) was found in guinea pigs and rabbits.

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DISCUSSION

If immersion of the whole animal in liquid nitrogen is considered as a base of reference without criticism, the values obtained for mice indicate that *in vivo*, ATP is by far the most abundant nucleotide (60 per cent of the total content of nucleotides), ADP is present in very small amounts, and AMP in traces. The ATP/ADP ratio for mice is 22. The distribution of the guanine nucleotides is similar, GTP being by far the most abundant with lower proportions of GDP and GMP; the GTP/GDP ratio is 10·7. For the uracil nucleotides the values decreased from 24·5 μmoles per 100 g to traces in the order UDPCo, UTP, UDP and UMP.

For mice immersed in liquid nitrogen, the values obtained were about the same whether or not the animals had been submitted to 1 min anaesthesia. This shows that results obtained using chloral hydrate are valid and that, at least with this anaesthetic, an accumulation of ATP or other nucleoside polyphosphates should not be expected.

For rat brains obtained by decapitation followed by freezing of the head in liquid nitrogen, our values for ATP were higher than those obtained by KORANSKY (1958). In addition, the ratios for ATP/ADP (9·4) and GTP/GDP (3·0) are appreciably higher than those reported previously (ALBAUM *et al.*, 1946; DOHRING and GERLACH, 1957; and KORANSKY, 1958). The degree of phosphorylation of adenine, guanine and uracil is therefore very high.

Analysis of the proportion of the nucleoside triphosphates within each group of nucleotides (Table 3) shows that, when conditions for the preparation of brain material are good, ATP represents 90 per cent of the total content of adenine nucleotides and GTP 88·2 per cent of the total guanine nucleotides.

The concordant results obtained in conditions which could be taken as a base of reference show that, *in vivo*, the free nucleotides are highly phosphorylated in mice as well as rats. Freezing of the head before the brain is dissected gives better results than the reverse procedure (Table 1). If the brain is frozen after dissection the values for the nucleoside triphosphates are considerably lower while those for the di- and monophosphates are higher. Furthermore, for rats immersed whole in liquid nitrogen, the values for nucleoside polyphosphates are lower than those obtained in conditions which we consider optimum, i.e. extremely rapid decapitation of normal or anaesthetized rats followed by freezing and dissection. It seems that sufficient time elapses during the freezing of the body of the rat for some degradation of highly phosphorylated nucleosides to occur.

As it was shown that rapid chloral anaesthesia did not cause an accumulation of polyphosphate nucleosides, the experiments on guinea pigs and rabbits were conducted using this method of anaesthesia followed by decapitation and immersion of the head in liquid nitrogen.

The results obtained show that the proportion of ATP is relatively lower for guinea pigs and rabbits than for rats and mice. We think that these are minimum values and that, *in vivo*, the proportion of ATP for rabbits and guinea pigs is higher than that found. There is much less doubt about the validity of the results for GTP and UTP for large animals. It will be noted that for rabbits and guinea pigs (Table 2) the ratio of GMP and UMP to GTP and UTP is lower than the ratio of AMP to ATP. This indicates that, even when the preparative conditions are not optimum, the degradation of guanine and uracil nucleoside triphosphates is less rapid than that of ATP. For large animals anaesthesia appears to be essential as is indicated by the results obtained on

TABLE 2.—FREE NUCLEOTIDES IN THE CEREBRAL HEMISPHERES IN DIFFERENT SPECIES

(μmoles per 100 g wet weight)

Nucleotides	Mouse*	Rat†	Guinea pig‡	Rabbit§
CMP	13·1	13·6	8·7	8·5
DPN	19·0	16·5	traces	traces
AMP	traces	3·2	37·3	21·1
ADP	8·6	15·0	49·3	36·3
ATP	184·0	141·3	88·2	111·5
GMP	traces	traces	4·8	2·2
GDP	2·8	8·3	13·0	10·8
GTP	30·0	25·4	16·3	19·4
UMP	traces	traces	3·9	2·8
UDP	3·8	3·0	traces	1·5
UDPCo	24·5	26·3	20·4	19·9
UTP	13·8	13·6	7·2	11·2
IMP	5·3	10·6	20·3	21·1

* Without anaesthesia, number of animals: 70
† With and without anaesthesia, number of animals: 33
‡ With anaesthesia, number of animals: 25
§ With anaesthesia, number of animals: 5

TABLE 3.—DISTRIBUTION OF THE VARIOUS NUCLEOTIDES AS PER CENT OF THE TOTAL QUANTITY OF NUCLEOTIDES WITH THE SAME BASE

Method	Adenine nucleotides (%)			Guanine nucleotides (%)			Uridine nucleotides (%)			
	ATP	ADP	AMP	GTP	GDP	GMP	UTP	UDPCo	UDP	UMP
Mice not anaesthetized plunged whole in liquid nitrogen	95·5	4·5	traces	91·0	8·9	traces	32·9	58·4	8·7	traces
Mice anaesthetized plunged whole in liquid nitrogen	94·5	4·0	1·5	91·0	9·0	traces	35·2	51·9	12·9	traces
Rats decapitated without anaesthesia, heads frozen in liquid nitrogen	88·5	11·3	0·2	69·4	30·6	traces	33·8	57·1	9·1	traces
Rats anaesthetized, decapitated, heads frozen in liquid nitrogen	90·0	5·2	4·8	88·2	11·8	traces	35·4	60·3	4·3	traces
Rats decapitated, brain dissected (20 sec) then frozen in liquid nitrogen	18·5	30·5	51·0	24·2	33·7	42·1	10·0	58·9	11·1	20·0
Rats plunged whole in liquid nitrogen	9·7	25·4	64·9	17·7	44·7	37·6	9·3	40·0	15·7	35·0
Rabbits decapitated without anaesthesia, heads frozen in liquid nitrogen	20·0	33·5	46·5	26·7	39·5	34·0	—	—	—	—
Rabbits anaesthetized, decapitated, heads frozen in liquid nitrogen	57·0	24·2	18·8	52·0	37·5	10·5	31·6	56·3	4·2	7·9

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rabbits decapitated without narcosis. The proportions of ATP, GTP and GDP are much lower than those obtained with narcosis or in conditions which can be regarded as a base of reference, such as the freezing of the whole body of a smaller animal. The time required to decapitate a rabbit is longer than for a rat and this tends to vitiate the results of the estimations of acid-soluble nucleotides.

Only small amounts of inosinic acid were found in mice and rats killed under conditions considered to be optimal. This should correspond to the state *in vivo*; appreciably higher quantities of IMP were found in guinea pigs and rabbits. We think this is an artefact due to deamination of adenine nucleotides.

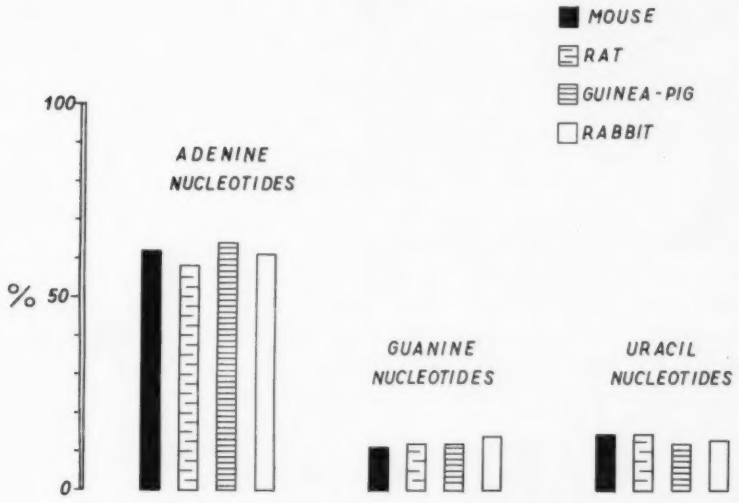

FIG. 5.—Percentage of the nucleotides of the same base in different species. Values in per cent of the total quantity of free nucleotides expressed in μmoles.

Except in young dogs we have not found CTP in determinable quantities. However, by using a large number of rat brains, we were able to show the presence of CDP-choline which participates in phosphatide synthesis (MANDEL, HARTH and REBEL, 1958). This is in agreement with a recent communication of ANSELL (1961). It should be noted that the values obtained for DPN are without doubt below those existing *in vivo* even for rats and mice. The degradation of this coenzyme in our preliminary procedure is so rapid that only a trace was found in guinea pigs and rabbits.

Differences in the actual amounts of adenine and guanine nucleotides for the various species are appreciable, whereas for the uridine coenzymes the divergence is less. However, the proportions of adenine, guanine and uracil nucleotides are similar in all the species. Those of adenine represent about 62 per cent of the total and those of guanine and uracil about 10–15 per cent (Fig. 5). It seems, therefore, that in this group of mammals there exists a certain similarity in the distribution of the free nucleotides of the brain.

It is concluded that brief anaesthesia induced by chloral hydrate does not modify the proportional distribution of the nucleotides in the cerebrum. For small animals immersed whole in liquid nitrogen similar results are obtained, whether or not they

have been anaesthetized. The same applies to normal or anaesthetized rats if rapid decapitation is followed by immediate freezing of the head. In these cases the proportion of triphosphate nucleosides is very high relative to di- and monophosphate nucleosides. The values are much higher than those published previously.

The freezing whole of an animal of the size of a rat causes a partial degradation of nucleoside polyphosphates.

In relatively large animals such as the guinea pig and rabbit killed after brief anaesthesia, the values for nucleoside triphosphates are much greater than those for di- and monophosphates. However, they seem to be less than those existing *in vivo*. The natural state of free nucleotides corresponds to one of high phosphorylation.

The UDPCo's seem to be stable and the values found depend much less on the preliminary procedure than do those of other nucleotides.

SUMMARY

The free nucleotides of the cerebral hemispheres of four species of mammals, killed in various conditions, were separated by ion-exchange chromatography and estimated quantitatively. Under conditions closest to the *in vivo* state the various types of nucleotides are essentially in the state of maximum phosphorylation. Very little nucleoside monophosphate is found.

ATP represents, in the mouse and rat respectively, 58 and 51 per cent of the nucleotides. The ratio ATP/ADP is 22 for the mouse and 9·4 for the rat.

A short anaesthesia with chloral hydrate does not appreciably modify the proportional distribution of nucleotides in rats or mice.

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INCORPORATION OF ^{32}P IN RIBONUCLEIC ACID OF SUBCELLULAR FRACTIONS OF VARIOUS REGIONS OF THE RAT CENTRAL NERVOUS SYSTEM

PAUL MANDEL, TOMASZ BORKOWSKI*, SUZANNE HARTH and RICHARD MARDELL

Institut de Chimie Biologique, Faculté de Médecine, Strasbourg, France and *Zaklad Chemii Fizjologicznej Akademii Medycznej, Lublin, Poland

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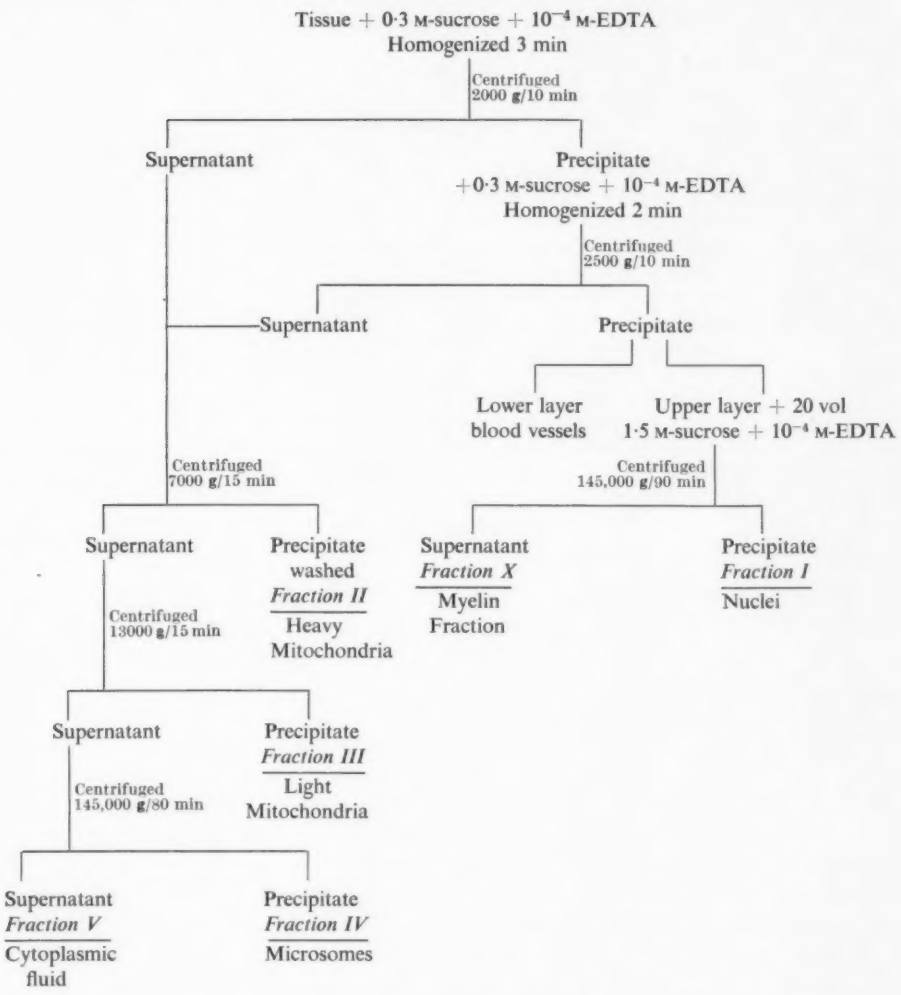
THE distribution of ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) in subcellular fractions of the whole brain of rat and rabbit was studied by BRODY and BAIN (1952). ABOOD, GERARD, BANKS and TSCHIRGI (1952) have given data on the distribution of nucleic acids and phospholipids in the cortex and white matter of the central nervous system of the adult rat and KEUP (1957) on the cortex of the rat brain during development. The results of BRODY and BAIN (1952) were confirmed by ALDRIDGE and JOHNSON (1959). Studies involving a supplementary fractionation have been made on subcellular fractions of whole brain (HANSON and TOSCHI, 1959; TOSCHI, 1959; DAHL, JACOBS and SAMSON, 1960; HEALD, 1959; JOHNSON, 1960). ROBERTSON (1960) studied the incorporation of ^{32}P in the phospholipids of subcellular structures of whole rat brain.

We showed previously that the RNA and DNA contents differ from one brain region to another. The same is true for the rate of incorporation of ^{32}P in RNA (MANDEL, HARTH and BORKOWSKI, 1960). These facts prompted us to study the distribution of the nucleic acids and the incorporation of ^{32}P in RNA of the subcellular fractions of various regions of the central nervous system (CNS): olfactory lobes, cerebral cortex, cerebral white matter, hypothalamus, cerebellum and medulla oblongata.

METHODS

In each of seven experiments, 20 adult albino rats of the Wistar strain, weighing 150–200 g were used. Each animal was given a single intraperitoneal injection of 200 μC of $H_3[^{32}P]O_4$ per 100 g body weight. After 1, 3 or 5 days, the rats were decapitated and the heads were immediately plunged into ice cold 0·9% sodium chloride solution. The brains were removed, the membranes were stripped off and the different parts were dissected out in a manipulation cabinet at 0° (MANDEL, 1959). Each part was washed in ice cold 0·9% sodium chloride and rapidly dried on filter paper before determination of the wet weight. Each specimen was homogenized in a mechanical grinder of the Potter type, for 3 min in 0·3 M-sucrose (4·3 ml per g wet weight), containing 0·1 mM ethylenediaminetetra-acetic acid. Half the quantity of the same sucrose solution was added to each homogenized specimen before differential centrifugation. Thus, initially, we followed the classical methods and separated in our first two experiments, fractions comprising nuclei, heavy and light mitochondria, microsomes and cytoplasmic fluid. However, examination of the nuclear fraction by phase contrast and electron microscopy revealed a considerable contamination by other cellular particles. We therefore modified the subcellular fractionation according to the following scheme.

THE SEPARATION OF SUBCELLULAR STRUCTURES OF THE CENTRAL NERVOUS SYSTEM



Fractionation. The 2·2 M-sucrose solution used by CHAUVEAU, MOULE and ROUILLER (1957) for the purification of liver nuclei did not permit adequate sedimentation of the nuclei of nervous tissue because of their lower density. A 1·5 M-sucrose solution was chosen after trials with different density gradients according to WHITTAKER (1959) and HEALD (1959). The subcellular fractions were examined each time by phase contrast and electron microscopy.

The acid-soluble material of each subcellular fraction was extracted with 0·6 N-perchloric acid at 0°. The lipids were extracted by treatment of the residue first with ice cold ethanol, then with ethanol–ether according to the method of BLOOR (1929). The lipid phosphorus content of the alcohol–ether extract was determined by the method of BRIGGS (1922) as modified by MACHEBOEUF and DELSAL (1943). In spite of the modification of the lipid constitution that this method causes we were able to verify that our extract contained at least 87 per cent of the lipid phosphorus obtained by the method of FOLCH, LEES and STANLEY (1957). In addition values for specific activity of the total lipid phosphorus were practically identical, whatever method of estimation was used. For this reason, we have continued to use the extraction procedure described above, which has the advantage that the same

sample can be used to determine lipid, nucleotide and nucleic acid phosphorus. In further work we shall study the incorporation of ^{32}P in phosphatides extracted according to the method of FOLCH *et al.* (1957). RNA and DNA were separated according to SCHMIDT and THANNHAUSER (1945). In order to provide RNA ribonucleotides exclusively the acid-soluble fraction of the alkaline hydrolysate was freed from non-nucleotidic compounds by a method in use in our laboratory (MANDEL, LEDIG and WEILL, 1958). Absorption at wavelengths of 2600 Å and 2860 Å (TSANEV and MARKOV, 1960) is determined on part of the acid-soluble fraction of the alkaline hydrolysate, which is then passed down a column of charcoal on which the ribonucleotides are adsorbed; the mineral phosphorus of phosphoproteins and the phosphorus of phosphopeptides originating from phosphatidopeptides remain in the effluent. The ribonucleotides of the RNA are eluted with a mixture of ethanol–ammonia–water (50 : 2 : 48). Provided the charcoal is suitably chosen and prepared, the elution is of the order of 90–95 per cent. The RNA content of the eluate is determined by absorption in the ultra-violet range combined with estimation of ribonucleic phosphorus.

The acid-insoluble fraction of the alkaline hydrolysate was heated twice with N-perchloric acid at 80° for 30 min according to SCHMIDT and THANNHAUSER (1945) to remove DNA. DNA was estimated by a combination of ultra-violet absorption (TSANEV and MARKOV, 1960) determination of phosphorus (MACHEBOEUF and DELSAL, 1943) and determination of deoxyribose according to the method of DISCHE (1930) as modified by BURTON (1956).

During the time required for subcellular fractionation, free nucleotides are hydrolysed to nucleoside monophosphates and nucleosides (HARTH and MANDEL, in press). It is therefore impossible to take much account of results of electrophoresis of the acid-soluble components. For this reason no results are reported.

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RESULTS

Method of fractionation

Results obtained by us (Table 1) using the classical method show that the nuclear fractions (I) of the six regions examined, contain 92·5–98·9 per cent of the total DNA, 41·1–65·2 per cent of the total RNA, 76–93·7 per cent of the total protein plus phosphopeptide phosphorus and 61·7–86·6 per cent of the total lipid phosphorus. Results obtained with the further purification of the nuclear fraction in our method of fractionation are given in Table 2. Excluding the results for the cerebellum, which were exceptionally high compared with the other regions, the nuclear fraction of the various parts contained 69–88·8 per cent of the total DNA, not more than 9–14·8 per cent of the total RNA, only 1·9–5·1 per cent of the total lipid phosphorus and 5–10 per cent of the total protein plus phosphopeptide phosphorus. The values for the nuclei of the cerebellum were 91·8 per cent of the DNA, 32·4 per cent of the RNA, 13·0 per cent of the lipid phosphorus and 20·8 per cent of the protein plus peptide phosphorus. By our method of fractionation, 38·6–71·0 per cent of the lipid phosphorus, 44·3–68·5 per cent of the protein plus peptide phosphorus were found in Fraction X, a fraction rich in myelin. The RNA content of the microsomes was 20·8–28·5 per cent of the total as determined by the classical method and 29·5–33·9 per cent as determined by the new fractionation procedure.

Distribution of phosphorus compounds in the subcellular fractions of various parts of the brain

Protein was taken as the base of reference for representing the quantities of lipid-P, RNA-P, DNA-P, and protein plus peptide-P in the subcellular fractions of the various zones.

The DNA/protein ratio varied from 6·3 to 14·3 for the nuclear fractions. For Fraction X, the values did not exceed 1·0. No measurable quantity of DNA was found in the other fractions.

TABLE 1.—DISTRIBUTION OF PHOSPHORUS COMPOUNDS IN SUBCELLULAR FRACTIONS PREPARED BY CLASSICAL METHODS

Results expressed as per cent of the total P estimated. Fractions: I Nuclear fraction, II Heavy mitochondria, III Light mitochondria, IV Microsomes, V Cytoplasmic fluid

Zones	Fractions	Phospholipid-P	Protein plus phosphopeptide-P	RNA-P	DNA-P
Olfactory lobes	I	71·20	76·00	41·10	96·6
	II	11·00	8·45	5·16	
	III	7·60	4·94	5·16	
	IV	8·63	4·82	27·10	
	V	1·58	4·83	21·54	
Cerebral cortex	I	77·20	89·00	43·80	93·7
	II	8·82	2·87	1·71	
	III	5·82	2·30	3·38	
	IV	7·00	2·75	27·50	
	V	1·17	3·15	23·50	
Cerebral white matter	I	86·60	90·90	50·70	95·6
	II	4·55	3·00	3·97	
	III	3·46	1·77	4·50	
	IV	4·69	2·22	20·80	
	V	0·70	2·19	19·99	
Hypothalamus	I	61·70	85·20	42·60	95·0
	II	13·80	9·30	6·40	
	III	11·54	—	6·70	
	IV	11.50	4·26	28·50	
	V	1·52	1·32	15·70	—
Cerebellum	I	85·40	91·60	65·20	98·9
	II	6·82	3·02	2·68	
	III	2·23	1·06	3·04	
	IV	4·48	2·68	21·90	
	V	1·04	1·60	7·07	
Medulla oblongata	I	86·60	93·70	56·80	92·5
	II	5·75	2·98	4·82	
	III	1·58	0·56	3·29	
	IV	4·67	1·92	24·30	
	V	1·45	0·91	10·70	

The RNA content of the hypothalamic nuclei was 1·69 μg P/mg protein. In other regions the values ranged from 0·91 to 1·27 μg. The cytoplasmic fluid (Fraction V) contained relatively little RNA, 0·65 to 1·44 μg P/mg protein. The highest values were obtained for the cortex and olfactory lobes. More RNA was present in the microsomes (Fraction IV) than in any other subcellular fraction, the figures ranging from 2·27 to 3·55 μg RNA-P/mg protein, being highest in the cortex and cerebellum. Fraction X, rich in myelin and containing axonal mitochondria, gave low values for RNA (0·29–0·57 μg RNA-P/mg protein). The values for the heavy mitochondria (Fraction II) ranged from 0·54 to 1·08 and for the light mitochondria (Fraction III) were fairly close to unity with a maximum of 1·40 for the olfactory lobes.

TABLE 2.—DISTRIBUTION OF PHOSPHORUS COMPOUNDS IN SUBCELLULAR FRACTIONS
Results expressed as per cent of each fraction in the whole tissue.
(Values in per cent of the total phosphorus)

Zones	Fractions	Phospholipid-P	Protein plus phospho-peptide-P	RNA-P	DNA-P
Olfactory lobes	I	5·1	10·0	14·8	88·8
	X	53·4	49·3	17·4	11·2
	II	14·3	10·7	6·8	
	III	10·2	8·5	6·9	
	IV	14·5	12·0	30·0	
	V	2·5	9·7	24·0	
Cerebral cortex	I	3·1	6·6	9·0	85·9
	X	55·2	57·2	15·8	14·1
	II	20·7	15·6	9·3	
	III	8·4	3·7	8·1	
	IV	11·2	7·4	33·9	
	V	1·2	9·4	24·0	
Cerebral white matter	I	2·6	4·8	10·3	71·5
	X	65·1	68·5	21·0	28·5
	II	12·9	11·5	9·7	
	III	5·9	3·2	7·3	
	IV	12·2	7·4	29·5	
	V	1·6	4·5	21·8	
Hypothalamus	I	3·4	7·8	10·0	74·6
	X	38·6	44·3	15·0	25·4
	II	24·7	16·6	12·1	
	III	11·2	8·2	10·1	
	IV	19·5	14·4	32·6	
	V	2·5	8·4	20·0	
Cerebellum	I	13·0	20·8	32·4	91·8
	X	55·3	52·9	15·4	8·2
	II	8·6	7·2	5·3	
	III	9·5	5·6	5·2	
	IV	11·7	9·1	31·0	
	V	1·8	4·5	10·6	
Medulla oblongata	I	1·9	5·0	9·3	69·0
	X	71·0	67·9	17·2	31·0
	II	9·6	11·3	16·1	
	III	5·6	4·2	10·9	
	IV	9·2	7·1	31·6	
	V	2·7	4·3	14·9	

Consideration of the results for protein plus peptide phosphorus reveals the interesting fact that, except for the olfactory lobes, the highest values were found in Fraction X (1·21 to 2·70) and the lowest in every case was in the cytoplasmic fluid (0·31 to 0·85). The average value for the microsomes was 1·17, for the light mitochondria 1·10 and for the heavy mitochondria 1·37 μg P/mg protein.

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TABLE 3.—RELATIONS BETWEEN VARIOUS COMPOUNDS AND PROTEIN CONTENT
(Values in μg P/mg protein)

Zones	Fractions	Phospholipid-P	Protein plus phospho-peptide-P	RNA-P	DNA-P
Olfactory lobes	I	8·0	0·91	1·27	13·6
	X	30·8	1·67	0·55	0·63
	II	37·9	1·64	0·99	
	III	37·7	1·83	1·40	
	IV	30·2	1·47	3·43	
	V	2·6	0·85	1·35	
Cerebral cortex	I	8·23	0·91	1·23	8·3
	X	22·9	1·21	0·35	0·22
	II	23·1	0·93	0·54	
	III	25·0	0·58	1·26	
	IV	22·5	0·82	3·55	
	V	1·45	0·57	1·44	
Cerebral white matter	I	7·96	0·92	1·05	8·6
	X	31·5	2·10	0·34	0·52
	II	22·3	1·25	0·55	
	III	24·7	0·85	1·00	
	IV	29·3	1·13	2·35	
	V	2·2	0·39	1·00	
Hypothalamus	I	12·0	1·52	1·69	8·6
	X	36·9	1·94	0·57	0·66
	II	29·0	0·89	0·56	
	III	28·0	0·94	1·01	
	IV	34·0	1·14	2·27	
	V	3·37	0·51	1·06	
Cerebellum	I	8·46	0·87	1·20	14·3
	X	31·1	1·91	0·49	1·0
	II	30·8	1·65	1·08	
	III	33·4	1·28	1·06	
	IV	23·1	1·16	3·49	
	V	2·0	0·31	0·65	
Medulla oblongata	I	9·12	1·27	0·98	6·3
	X	52·9	2·70	0·29	0·44
	II	29·4	1·85	1·01	
	III	27·6	1·13	1·21	
	IV	32·3	1·33	2·45	
	V	5·63	0·48	0·68	

An examination of the lipid-P content of the nuclear fractions shows a maximum of 12·0 μg P/mg protein for the hypothalamus and 7·96 to 9·1 μg for the other zones. For the cytoplasmic fluid the lowest content was found in the cortex (1·45 μg P) and the highest in the medulla (5·63 μg). The myelin-rich Fraction X contained 22·9–36·9 μg lipid-P/mg-protein with the exception of the medulla which was noticeable for its high value of 52·9 μg. For both mitochondria and microsomes the values within

each zone are of about the same order of magnitude, and range from 22·5 to 37·7 μg.

The RNA/DNA ratio of the nuclei (Table 4) varies from a minimum of 0·08 for the cerebellum to a maximum of 0·178 for the hypothalamus. Recently, GEORGIEV and MANT'EVA (1960) isolated nuclei from whole brain by the phenol method and found an RNA/DNA ratio of 0·117. In our opinion the microscopic appearance of the phenol preparations was not satisfactory. It is interesting to recall that CHAUVEAU *et al.* (1957) found a value of 0·16 for purified liver nuclei. The ratios for the nuclear lipid and protein plus peptide phosphorus are highest for the cerebral white matter, the hypothalamus and the medulla.

TABLE 4.—RATIO OF VARIOUS COMPOUNDS TO DEOXYRIBONUCLEIC PHOSPHORUS IN THE NUCLEAR FRACTION

(Values expressed in μg/μg DNA-P)

Zones	RNA-P	Protein plus phosphopeptide-P	Phospholipid-P	Proteins
Olfactory lobes	0·105	0·067	0·665	74
Cerebral cortex	0·166	0·110	0·890	120
Cerebral white matter	0·124	0·108	1·159	118
Hypothalamus	0·178	0·176	1·140	118
Cerebellum	0·082	0·062	0·533	70
Medulla oblongata	0·154	0·194	1·159	153

Incorporation of $H_3[^{32}P]O_4$

During the first 3 days after injection of $H_3[^{32}P]O_4$ (Table 5) the RNA specific activity was higher in the nuclear fraction of each zone than in the other subcellular fractions, being highest in the olfactory lobes and hypothalamus. After 5 days the specific activity of the nuclear fraction of the cortex, white matter and medulla was still increasing and in these regions was still higher in the nuclear fraction than in the other subcellular fractions. The specific activity of the nuclear fraction of the olfactory lobes, cerebellum and hypothalamus was decreasing after 5 days and at that time in these regions was highest in the cytoplasmic fluid.

A study of the incorporation of ^{32}P in RNA as a function of time for the different regions of the central nervous system (Fig. 1) shows that uptake is most rapid in the hypothalamus followed by the medulla, the olfactory lobes and the cerebellum. The highest values attained for the specific activity of the cytoplasmic fluid, which contains soluble RNA, are for the hypothalamus and olfactory lobes, followed by the cerebellum and medulla. For the microsomes, the specific activity is highest in the olfactory lobes and next highest in the hypothalamus, cerebellum and medulla which all reach about the same level.

The data presented here concerning the velocity of incorporation of ^{32}P in the RNA of the subcellular fractions of the various zones are in good agreement with the

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results obtained for the same zones considered in their entirety (MANDEL *et al.*, 1960).

The incorporation of ^{32}P in the phospholipids (Table 6) followed the same pattern in distribution for the various subcellular fractions and increased and decreased with time in the same manner as for RNA, with the exception of the cerebellum. Here the activity of the phospholipids continued to increase in the nuclei during the 5-day period and was higher there at all times than in the other subcellular fractions of the cerebellum.

TABLE 5.—SPECIFIC ACTIVITIES OF RNA IN THE DIFFERENT SUBCELLULAR STRUCTURES OF VARIOUS ZONES OF THE RAT CENTRAL NERVOUS SYSTEM
(Counts/min/μg P)

Zones	Hr	Nuclei	Myelin fraction	Heavy mitochondria	Light mitochondria	Endoplasmic reticulum	Cytoplasmic fluid
Olfactory lobes	24	37	36	24	10	20	33
	72	75	49	39	40	51	57
	120	52	54	—	—	68	69
Cerebral cortex	24	34	24	9	16	9	13
	72	43	24	29	25	23	30
	120	57	27	32	30	40	41
Cerebral white matter	24	26	17	7	8	8	13
	72	46	29	26	21	27	32
	120	49	35	30	28	37	39
Hypothalamus	24	58	34	24	11	30	19
	72	64	47	36	38	44	47
	120	51	29	51	42	51	88
Cerebellum	24	39	—	9	9	14	16
	72	58	47	34	31	35	47
	120	57	46	—	—	46	63
Medulla oblongata	24	50	28	9	18	15	13
	72	56	45	30	38	36	38
	120	71	40	—	—	53	39

Fraction X contained myelin and axonal mitochondria. A special study will be made of the constitution and renewal of the purified structures in Fraction X. A consideration of the proportion of the activity of these structures in the overall specific activity shows that that of the myelin sheaths is the lowest of all the subcellular fractions.

DISCUSSION

Method of fractionation

Using our method of fractionation, it was found that the nuclear fraction contained a lower proportion of the total DNA phosphorus and much lower proportions of the RNA, lipid, and protein-peptide phosphorus than were obtained by classical methods. The differences were accounted for mainly by the amounts contained in the new Fraction X, although the values, obtained by our method, for RNA, lipid, and protein-peptide phosphorus were also higher in the mitochondria and microsomes than those

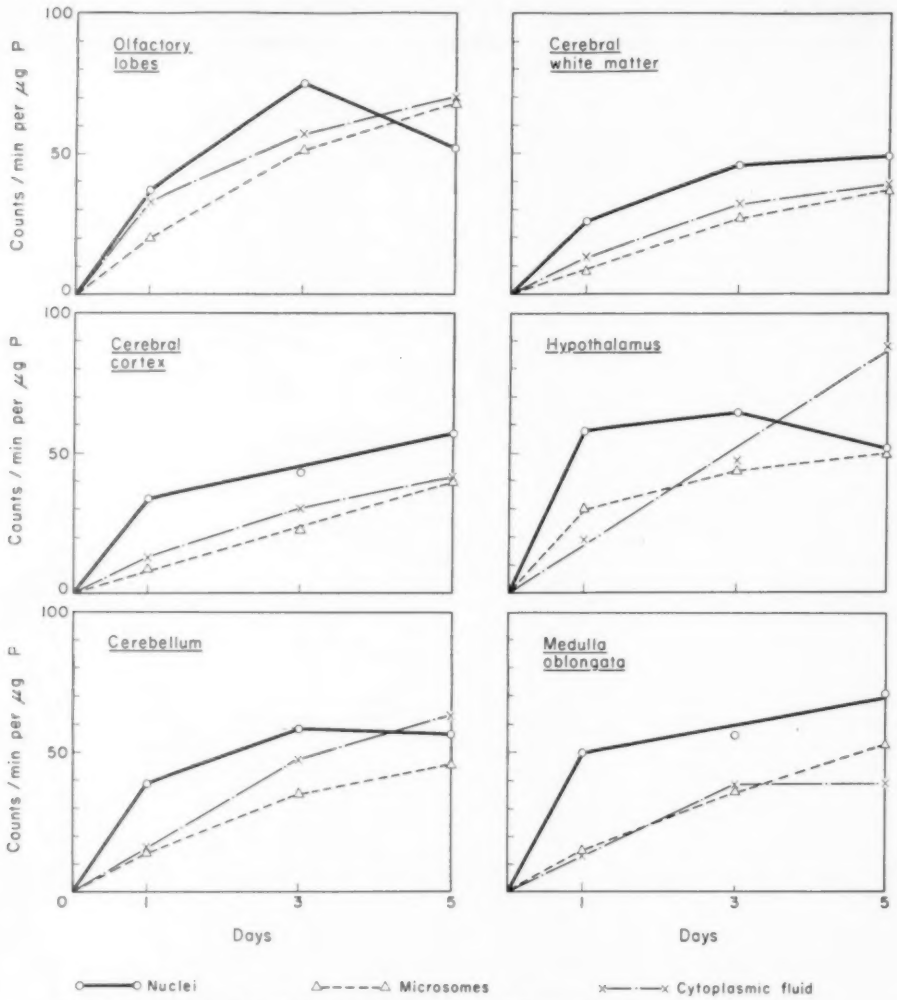

FIG. 1.—Specific activity of ribonucleic phosphorus of three subcellular fractions of rat brain. Values expressed in counts per min per μg P at various times after intraperitoneal injection of 200 μC $H_3[^{32}P]O_4$ per 100 g body weight.

obtained by the classical procedure. The RNA/DNA ratio of the nuclear fraction we isolated was 0·129–0·169 compared with the 0·36 value obtained from the data of ALDRIDGE and JOHNSON (1959), 0·7–1·1 given by ABOOD *et al.* (1952) and 1·2 given by BRODY and BAIN (1952).

Fraction X isolated by purification of the classical nuclear fraction was shown by electron microscopy and chemical and enzymic estimations to contain myelin sheaths and mitochondria.

The ribonucleic acids and proteins

As expected, the highest values for RNA, expressed in μg phosphorus/mg protein, were obtained for the microsomal fractions. In these fractions important differences

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exist between one region of the central nervous system and another, with lower values being obtained for white matter, the hypothalamus and the medulla. The RNA/protein ratio for the cytoplasmic fluid was highest for the cortex and the olfactory lobes. This indicates a greater abundance of soluble RNA, which, as is known, participates in the transfer of amino acids and in consequence, in the synthesis of proteins.

TABLE 6.—SPECIFIC ACTIVITIES OF PHOSPHOLIPIDS IN DIFFERENT SUBCELLULAR STRUCTURES OF VARIOUS ZONES OF THE RAT CENTRAL NERVOUS SYSTEM
(Counts/min/μg P)

Zones	Hr	Nuclei	Myelin fraction	Heavy mitochondria	Light mitochondria	Endoplasmic reticulum	Cytoplasmic fluid
Olfactory lobes	24	81	28	37	34	55	47
	72	118	59	58	65	80	73
	120	74	46	—	—	76	81
Cerebral cortex	24	67	12	16	19	15	23
	72	69	31	37	36	35	38
	120	77	33	41	40	52	47
Cerebral white matter	24	52	8	14	14	16	15
	72	59	21	30	32	27	32
	120	77	23	34	39	31	36
Hypothalamus	24	71	16	26	26	39	31
	72	76	37	43	46	62	49
	120	40	35	51	42	49	51
Cerebellum	24	45	17	26	28	26	25
	72	71	39	62	63	51	61
	120	78	47	—	—	54	64
Medulla oblongata	24	—	6	18	20	22	28
	72	65	22	44	53	40	51
	120	88	28	—	—	55	53

It is interesting to examine the high values for the RNA/DNA ratio of the nuclei of the cortex and hypothalamus. It is known that neurons represent only a small fraction of all the cells of each zone, the majority being glial cells, of which the RNA/DNA ratio probably lies between 0·124 and 0·08, the values we obtained for the white matter and the cerebellum respectively. It is concluded that the quantity of RNA in the nuclei of the cortex, hypothalamus and medulla is particularly high. The high proportion of phosphopeptide phosphorus in the nuclei of the hypothalamus and medulla should also be noted.

The RNA/protein ratio is of the same order for the nuclei of all the regions except the hypothalamus, where the ratio was higher (1·69). This is another indication of the relative richness in RNA of the nuclei of the hypothalamus.

The protein content of the nuclei, as shown by the ratio protein/DNA, varies according to the zone. The nuclei of the hypothalamus and medulla are among the

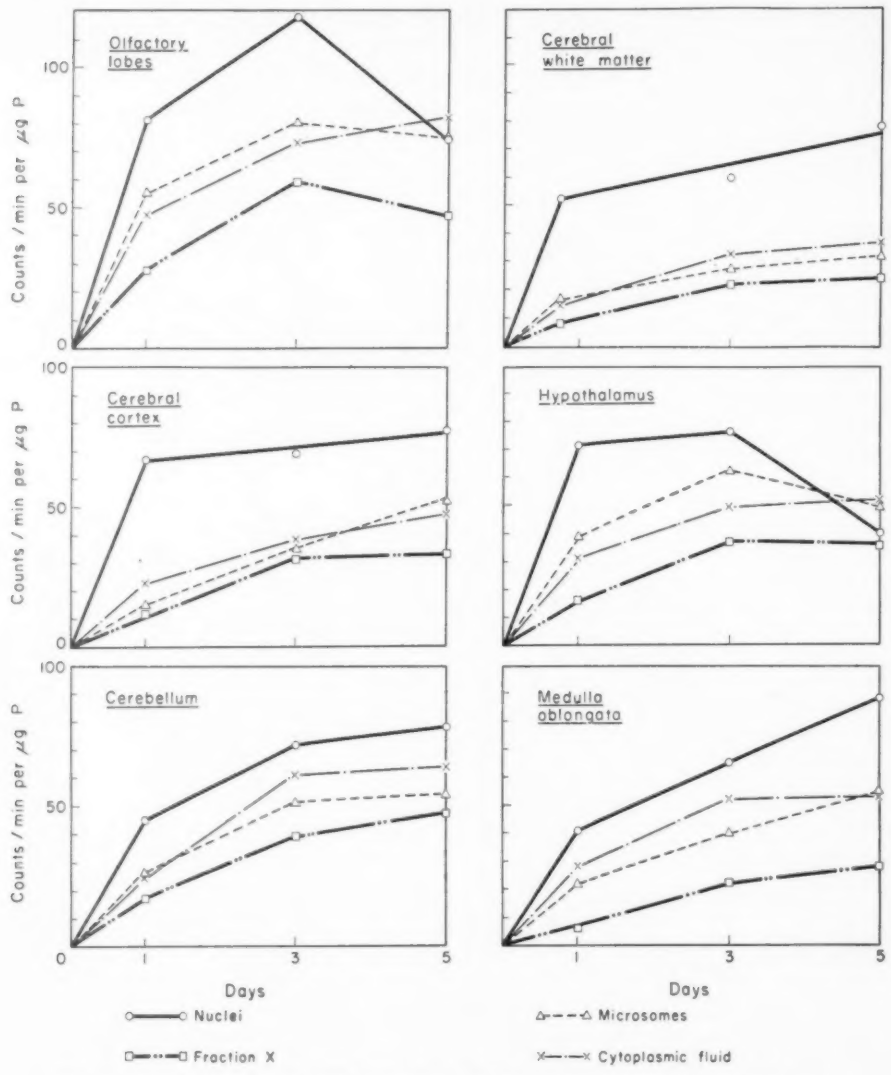

FIG. 2.—Specific activity of lipid phosphorus of four subcellular fractions of rat brain. Values expressed in counts per min per μg P at various times after intraperitoneal injection of 200 μC $H_3[^{32}P]O_4$ per 100 g body weight.

richest in protein, those of the olfactory lobes and the cerebellum being among the poorest. This is in agreement with histological information.

Protein and phosphopeptide phosphorus

Phosphopeptide phosphorus accounts for about 90 per cent of the total protein phosphorus plus peptide phosphorus originating from phosphatidopeptide (LEDIG and MANDEL). The values obtained for the two types of phosphorus compounds together therefore depend mainly on the phosphatido-peptide content. It is interesting to note

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in this connection that Fraction X is richest in these compounds. However, appreciable quantities are also found in the mitochondria and nuclei. The lowest values are for the cytoplasmic fluid. For a particular fraction, the figures vary according to zone between fairly wide limits indicating differences in the constitution of the subcellular particles. For example, the nuclei of the medulla and hypothalamus contain 1·27 and 1·52 mg protein plus phosphatidopeptide phosphorus per mg protein, whereas the other nuclei contain between 0·87 and 0·92 μg.

Incorporation of ^{32}P

The rapid incorporation of ^{32}P in the hypothalamus, olfactory lobes and cerebellum is in good agreement with the results we obtained previously for these zones considered in their entirety (MANDEL *et al.*, 1960). The rapid increase in the specific activity of the hypothalamus may be due to the rapid diffusion of ^{32}P in this zone. However, the fact that in this region the decrease in activity of the RNA of the nuclear fraction takes place between the third and fifth days and that the soluble RNA reaches particularly high values, indicates a rapid renewal of RNA which is, without doubt, related to the intensity of protein synthesis in the zone. The maintenance of a high specific activity in the cell nuclei for a long period distinguishes the cells of the central nervous system from those of other organs such as the liver, the kidney or the spleen, in which, during the first few hours after ^{32}P injection, the specific activity increases rapidly and then decreases just as quickly (WEILL and LEDIG, 1957; AMANO and LEBLOND, 1960; DAVIDSON, 1957).

The incorporation of ^{32}P in the phosphatides is similarly highest in the nuclear fraction during the first 3 days after injection of the isotope. As in the case of RNA renewal, the most rapid incorporation in the phosphatides is found in the nuclear fraction of the hypothalamus and olfactory lobes. The specific activities of lipid phosphorus differ in the subcellular fractions of the various zones, indicating, as for RNA, a certain biochemical autonomy.

The maintenance of the radioactivity of the phosphorus compounds over a long period should not, we think, be attributed to a slow turnover. There is, in fact, a discrepancy between the rate of entry and the rate of loss of ^{32}P for the different phosphorus compounds we have studied. We think that the explanation of this phenomenon is that the radioactive compounds which leave the constituents of high molecular weight remain in the pool of precursors of each zone and are utilized again. This is evidently a consequence of the structure of the extracellular system of the central nervous system which communicates very much less with the exterior than in other organs, thus preventing the diminution of the radioactivity of the acid-soluble compounds. In short, the maintenance of high radioactivity in the RNA, phosphoproteins and phosphatides or phosphopeptides is due to a continual re-use of radioactive compounds issuing from cellular constituents.

SUMMARY

A method of subcellular fractionation was developed, permitting the isolation of pure nuclei and myelin sheaths from the olfactory lobes, cerebral cortex, white matter, hypothalamus, cerebellum and medulla oblongata of the rat. Study of the distribution and renewal of ribonucleic lipid- and phosphoprotein-phosphorus of six subcellular fractions showed considerable differences among the zones examined. The cell

nuclei of the hypothalamus are among the richest in ribonucleic acid, protein and other phosphorus compounds. ^{32}P is incorporated most rapidly in the ribonucleic acid and phospholipid of cell nuclei which maintain a high radioactivity for a long time. The results support the existence of a certain metabolic autonomy in the various regions of the central nervous system.

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THE QUANTITATIVE HISTOCHEMICAL DISTRIBUTION OF THIAMINE IN DEFICIENT RAT BRAIN*

P. M. DREYFUS†
The McLean Hospital Research Laboratory, Waverley, Massachusetts,
and the Department of Neurology and Psychiatry (Neuropathology),
Harvard Medical School, Boston, Massachusetts.

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THE dietary deprivation of thiamine in both humans and experimental animals results in a number of characteristic neurological manifestations, many of which can be promptly reversed by the administration of either the coenzyme (thiaminepyrophosphate) or the free vitamin (thiamine). The histopathological alterations resulting from the lack of thiamine characteristically involve certain parts of the neuraxis in a selective and bilaterally symmetrical fashion (PRICKETT, 1934; KALM, LUCKNER and MAGUN, 1952; DREYFUS and VICTOR, 1960). Although the general histological appearance of these changes is very similar from species to species, different anatomical sites are usually affected (DREYFUS and VICTOR, 1960). In the rat, a bilaterally symmetrical area of pannecrosis is frequently observed in the vicinity of the lateral vestibular nucleus situated in the lateral pontine tegmentum. The afflicted portions of the brain thus exhibit what appears to be a selective vulnerability to the lack of the cofactor.

The demonstration of a striking 'biochemical lesion' in avitaminous pigeon brain by PETERS (1936), the relatively restricted and localized nature of the biochemical changes (KINNERSLEY and PETERS, 1930; GAVRILESCU and PETERS, 1931; MEIKLEJOHN, PASSMORE and PETERS, 1932) and the relation of these abnormalities to the symptoms and signs of thiamine deficiency have provided the stimulus for further investigations on this subject.

In an attempt to clarify further the problem of selective vulnerability, a series of investigations on various aspects of thiamine deficiency and its effects on the nervous system have been undertaken in this laboratory. As a first step, the distribution of thiamine was determined in various subdivisions of the nervous system of normal animals. It was hoped, thereby, to demonstrate differences in the vitamin content of the more vulnerable parts of the brain. Such differences would have partially explained the inherent selectivity of the pathological process. The results of the study (DREYFUS, 1959) revealed that total thiamine is evenly distributed throughout the brain with the exception of the cerebellum and the thalamus, the former containing more and the latter less vitamin than the remainder of the brain. Since no correlation could be made between tissue thiamine levels in the normal brain and the susceptibility of certain parts of the neuraxis to vitamin B_1 deprivation, it was decided to undertake a similar survey in animals at various stages of depletion. These experiments represent a pilot

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† Formerly, Special Trainee of the National Institute of Neurological Diseases and Blindness, U.S. Public Health Service, 1957–1958.

study designed to correlate pathological alterations induced by the deficiency state with thiamine levels in various anatomical subdivisions of the nervous system. Furthermore, it was hoped to demonstrate possible differences in the rate or extent of vitamin depletion of these same parts. It is the purpose of this communication to report the results of these experiments.

MATERIALS AND METHODS

Female rats 4–6 months of age were used for this series of experiments. The animals were bred in the laboratory from a Wistar type strain generously supplied by the Merck Institute of Therapeutic Research, Rahway, N.J. All of the animals were fed a synthetic thiamine-deficient diet. The diet was supplied by the Nutritional Biochemicals Corporation. It had the following composition (unit weight per 10 kg of diet): vitamin-free casein, 2000 g; sucrose, 7100 g; vegetable oil, 400 g; cod liver oil, 100 g; salt mixture (Hegsted) 400 g. To this basic mixture the following were also added: choline chloride, 4 g; riboflavin, 112 mg; calcium pantothenate, 280 mg; niacin, 560 mg; pyridoxine-HCl, 56 mg; folic acid, 14 mg; menadione, 14 mg; vitamin B_{12}, 180 mg. The thiamine content was adjusted to give 0·1 mg/kg of diet (1 mg of thiamine/kg of diet is required for normal growth and development). The clinical course of the animals was carefully followed. Each animal was weighed every two days during the first two weeks of the experiment, and then daily after the first signs of deficiency were detected. The animals were killed by decapitation at various stages of depletion and replenishment (Fig. 1). The brain, spinal cord, and sciatic nerve were removed and frozen immediately in a dry-ice chest.

The tissue-sampling technique employed in these experiments was essentially as previously described for normal animals (DREYFUS, 1959). Cerebral cortex, caudate nucleus, thalamus, hypothalamus, mammillary region, periaqueductal region, lateral pontine tegmentum, base of pons, cerebellar vermis, and medulla were sampled by means of a metal cork-borer 2 mm in diameter. The cylinder thus obtained was cut serially into 22 sections 40 μ in thickness with a rotary microtome. Sections 1, 8, 15, and 22 were used as histological controls. These sections were placed on an albuminized slide, thawed, fixed in alcohol–formalin, rinsed in distilled water, and strained overnight with buffered thionin (WINDLE, RHINES, and RANKIN, 1943). The remaining 18 sections were used for thiamine assay. Pooled groups of six consecutive sections were desiccated over calcium chloride at $-12°$ for 2 days and at room temperature for 3–5 days. This method of desiccation did not alter the thiamine content of the samples. The pooled sections were weighed and placed in a micro-homogenizer for thiamine determination. Each pooled sample weighed 150–220 μg. Entire cross-sections of the cervical portion of the spinal cord (300–450 μg) and small samples of sciatic nerve (0·5–1·0 mg) were also used for chemical analysis.

The method employed for total thiamine estimations was identical with the one described for normal material (DREYFUS, 1959). In brief, this method consists of homogenization of the samples in glass redistilled water and inactivation of all existing enzymes by heat. Protein is precipitated with trichloroacetic acid, and phosphorylated thiamine is converted to free thiamine by enzymic hydrolysis with a human prostatic acid phosphotase preparation. Oxidation of thiamine to thiochrome is accomplished by the use of alkaline potassium ferricyanide. This is followed by the extraction of thiochrome into *n*-hexanol, and measurements of fluorescence before and after exposure of the samples to an ultraviolet light source.

Neuropathological observations were made on other animals rendered thiamine deficient in the same manner as those used in the aforementioned experiments. Samples of the central nervous system were fixed in 10% formalin and embedded in celloidin. Semi-serial coronal and sagittal sections were stained for cellular structures with a Nissl stain, and medullated elements were stained by the Loyez method. (A detailed communication dealing with pathological observations will be made separately).

RESULTS

A representative weight curve of a female rat during thiamine depletion and replenishment is shown in Fig. 1. Each lettered point corresponds to a particular clinical stage. At point A the animal is normal. After approximately two weeks on the deficient diet, the animal ceases to gain weight; a decrease in spontaneous activity

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and appetite is noted (point B). This state is followed by a 2–3 week period of steady weight loss. At point C the animal is slow. At D it begins to show some unsteadiness of gait; posture is frequently abnormal, the back being arched. At times the animal tends to walk backwards or in circles, pivoting around its forelegs. Point E marks the point of maximal neurological impairment. The animal displays a severe disturbance of posture and equilibrium, as well as severe anorexia, looseness of fur, and varying degrees of hindquarter weakness. These neurological signs progress rapidly, and death

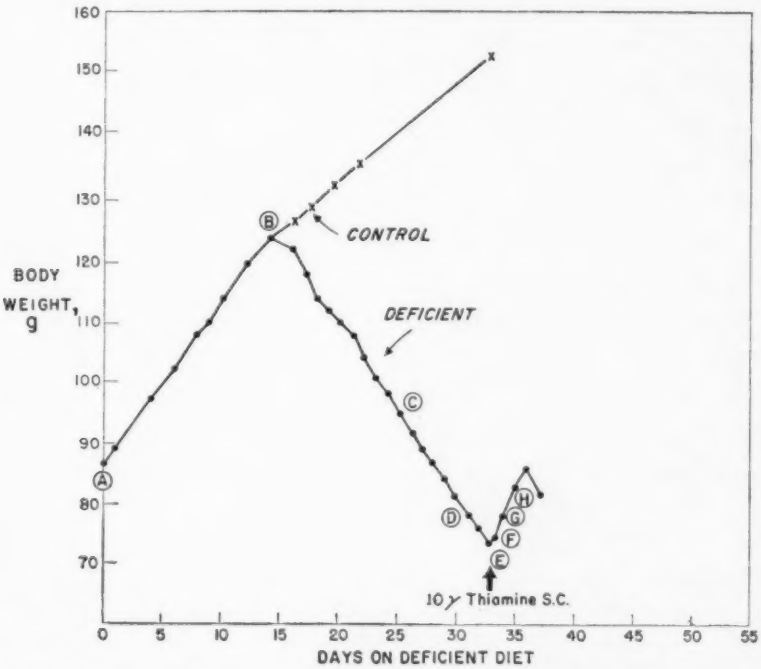

FIG. 1.—The effect of thiamine deficiency on the growth of rats. The line marked 'control' shows the rate of growth of animals which received a synthetic diet containing 4–5 mg of thiamine per kg of diet. Between points A and B normal and deficient animals showed no appreciable differences in rate of growth.

ensues within 24 hr unless the animal receives specific treatment. Two hours after the subcutaneous injection of 10 μg of thiamine into a severely depleted animal (point F) no changes can be noted in the animal's behaviour. A day later (point G) marked improvement has taken place: the animal has gained in weight, only slight ataxia is visible, and appetite is restored to normal. Two days after the injection (point H) the animal is nearly normal in its behaviour, except for some degree of slowness and residual hindquarter weakness.

Pathological examination of the nervous system of deficient animals discloses a number of abnormalities. Prominent astrocytic hyperplasia in many of the brain-stem and cerebellar nuclei (dentate, cochlear, facial, trigeminal, and red nuclei) is present in all of the animals. Nearly all show discrete, bilaterally symmetrical changes of varying degree and extent in the dorsal pontine tegmentum, in the region of the lateral vestibular nucleus. The most severe lesions, found in about one-third of the animals,

consist of a discrete, symmetrical area of tissue destruction and marked glial proliferation (Figs. 2 and 3). Many neurones are in varying stages of destruction. Within the centre of such a lesion all medullated fibres are destroyed. The more subtle changes observed in this region are characterized by astrocytic hyperplasia and proliferation as well as neuronal changes, the medullated fibres remaining intact.

TABLE 1.—DISTRIBUTION OF TOTAL THIAMINE IN THE NERVOUS SYSTEM OF NORMAL AND DEFICIENT RATS AT VARIOUS POINTS OF DEPLETION

State of deficiency	Thiamine content (μg/g dry wt)								
	Normal‡		Deficient						
	A*		B	C	D	E	F	G	H
Number of animals	8		2	1	2	4	1	1	1
Area sampled									
		S.D.							
Cortex	13·0	±2·5	7·6	4·8	4·4	2·1	3·0	3·8	3·8
Caudate nucleus	17·4	±4·5	7·8	5·3	3·1	2·8	3·3	5·5	4·8
Thalmus	9·2	±2·3	6·3	4·8	5·6	2·1	1·8	2·7	3·2
Hypothalamus	13·0	±3·2	6·6	6·4	5·7	3·1	3·3	4·0	4·8
Mammillary region	12·4	±2·4	6·1	4·9	3·7	2·5	3·0	5·7	4·5
Periaqueductal region	13·2	±3·5	6·9	4·5	3·1	2·4	2·3	4·0	4·0
Lateral pontine tegmentum	11·5	±4·7	6·3	3·9	3·5	1·8	3·0	3·9	2·5
Base of pons	13·1	±2·5	5·4	3·4	2·3	2·3	2·5	2·7	2·1
Vermis	21·1	±4·2	5·4	3·8	4·1	2·3	3·3	3·3	2·7
Medulla	13·4	±3·1	6·2	3·6	3·6	2·4	2·5	3·2	2·6
Total brain†	13·7	±1·2	6·4	5·4	3·9	2·4	2·8	3·9	3·5
Spinal cord	8·4	±2·4	3·3	2·7	2·1	1·6	1·5	2·2	1·6
Sciatic nerve	4·1	±0·9	1·4	1·0	1·7	1·0	0·8	0·9	1·8

Each value is the average of triplicate determinations.
* Letters correspond to lettered points of curve in Fig. 1.
† Total brain values represent an average figure of the ten areas of the brain sampled.
‡ Values for normal brain, spinal cord and sciatic nerve are taken from previously published experiments (DREYFUS, 1959).

The results obtained from total thiamine determinations (Table 1) show that the rate and extent of total thiamine depletion exhibited by the various areas of the nervous system sampled are relatively uniform. Extreme depletion (point E, Fig. 1) results in a total brain thiamine loss of 82·5 per cent. The loss of cofactor in the various parts sampled is remarkably uniform, ranging from 89 to 76 per cent. The rate of replenishment of thiamine after the subcutaneous administration of 10 μg of the vitamin into severely depleted animals is difficult to evaluate in view of the methodology employed.

DISCUSSION

The results obtained from the foregoing series of experiments provide further information regarding the content of total thiamine in various subdivisions of the

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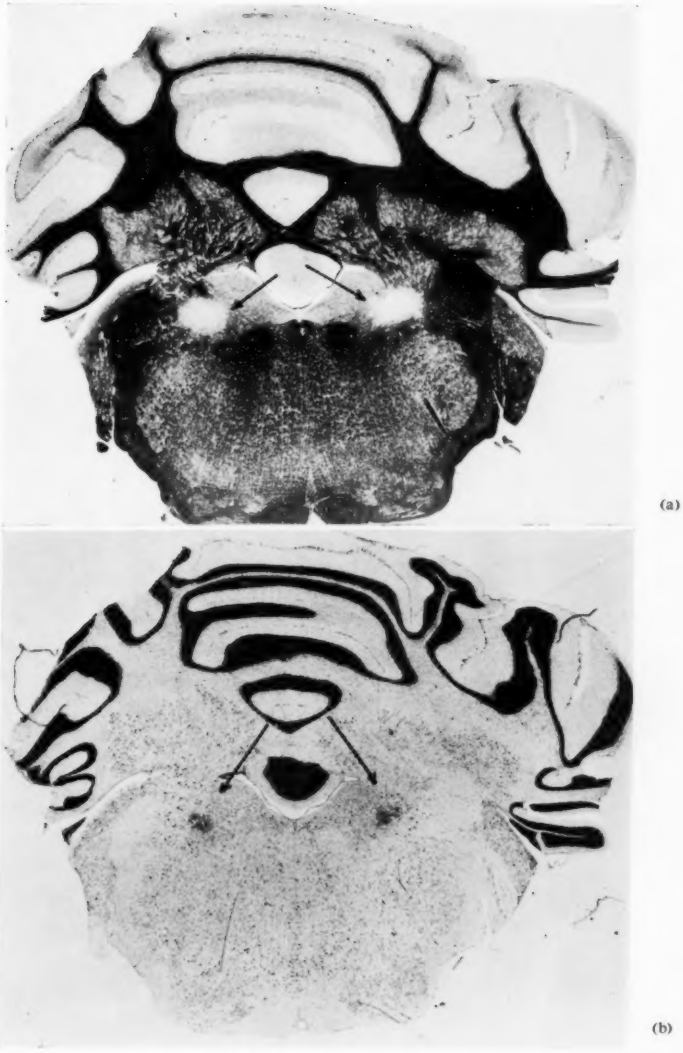

FIG. 2.—Sections through caudal pons showing bilaterally symmetrical lesions (arrows) in the vicinity of the lateral vestibular nucleus. (a) Loyez myelin stain, showing destruction of medullated structures. (b) Cresyl violet stain, showing proliferation of glial cells.

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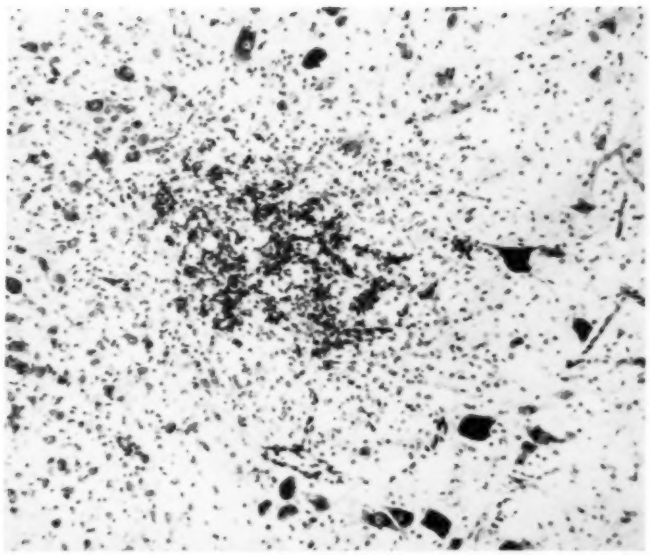

FIG. 3.—Microscopic details of a characteristic lesion in the lateral pontine tegmentum showing proliferation of histiocytes, macrophages and astrocytic hyperplasia. Nerve cells are relatively preserved.

nervous system at different stages of vitamin depletion. Although the results fail to shed further light on the apparent selectivity of certain anatomical sites to the effects of thiamine deprivation, a number of general remarks concerning this subject can be made.

The relationship between the clinical appearance of the animals on the one hand and total brain thiamine on the other (Fig. 4) shows that total brain thiamine can be reduced to about 50 per cent of normal before any clinical signs of deficiency become manifest. A further decrease to 30 per cent of normal is accompanied by slowness and

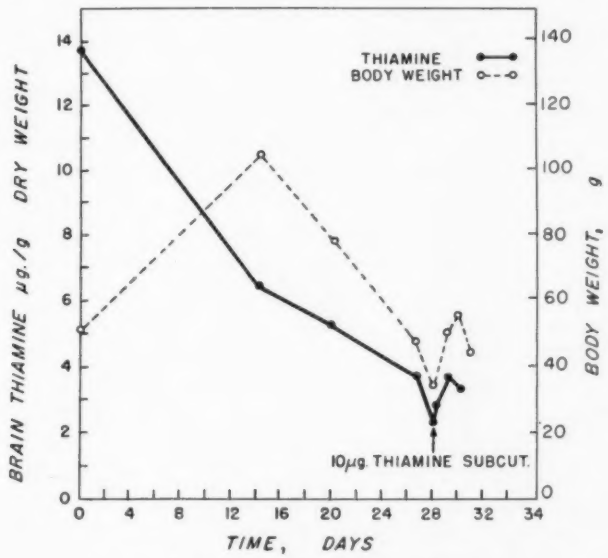

FIG. 4.—Brain thiamine in progressive depletion.

unsteadiness of gait. When brain thiamine is reduced further to less than 20 per cent of normal, a severe disturbance of posture and equilibrium occurs, and these are accompanied by irreversible pathological changes in most of the animals. If, at this point, 10 μg of thiamine are injected subcutaneously, the level again rises to 30 per cent of normal after 24 hr, and the symptoms are dramatically improved. After 48 hr the thiamine level again falls to less than 30 per cent, yet no worsening of symptoms can be noted.

A number of factors undoubtedly affect the results of these experiments. Although the sampling procedure is relatively reproducible from one experiment to the next, in terms of anatomical subdivision, the overall histological composition of each sample varies considerably. It is conceivable that if one were able to follow the thiamine content of glial elements and neurones separately in the various anatomical sites, differences could indeed be demonstrated. Such a tedious sampling procedure did not seem practical considering the fact that we have not yet established which of the elements of the nervous system are primarily affected by the deficiency state (glia, neurones, or medullated fibres).

A comparison of the total thiamine content in various parts of the nervous system of different animals at various stages of depletion and replenishment is rendered

difficult by virtue of the existing wide range of tissue vitamin levels in normal animals. Attempts to correlate the severity of the irreversible histopathological changes in the lateral pontine tegmentum and the vitamin content in this part of the brain have failed. It is very likely that the critical tissue thiamine level responsible for severe pathological alterations differs from animal to animal. The vitamin content in itself is probably only one factor in the causation of irreversible central nervous system lesions. If one were able to maintain the animals at extremely low thiamine levels for long periods of time the histopathological changes would undoubtedly be more spectacular in each instance. Unfortunately, sudden death from cardiac failure often occurs before the pathological changes in the nervous system become manifest. This fact has been commonly observed in human beriberi (DENNY-BROWN, 1958).

The experimental data suggest that in rat brain as in pigeon brain (OCHOA and PETERS, 1938) there exists a considerable 'surplus' of thiamine pyrophosphate (cocarboxylase) which provides a wide margin of safety against the adverse effects of deficiency. Whether all of the excess cocarboxylase is of metabolic value cannot be stated with certainty. In pigeon heart slices OLSEN and his coworkers (1948) observed a depression of pyruvate oxidation when the thiamine content fell below 25 per cent of normal. This suggests an inactive surplus of coenzyme. Other enzyme systems known to require cocarboxylase as cofactor (α-ketoglutaric oxidase and transketolase) have as yet not been studied in a similar fashion.

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Since both coenzyme and apoenzyme are necessary for optimal enzymic activity it is conceivable that the selective vulnerability of certain parts of the neuraxis is in some way related to an abnormality of ratio of one to the other. Thiamine deficiency results in a gradual depletion of coenzyme. If depletion is allowed to progress to extreme levels there may result, in addition, a depletion of apoenzyme. Although such a depletion has not been demonstrated as yet, the thiamine deficient state appears to be attended by demonstrable abnormalities in protein metabolism. Thus, JIMENÉZ-DIAS *et al.* (1947) showed depletion of total protein in the carcasses of thiamine deficient rats. A defect in amino acid metabolism is suggested by the observations of DALGLIESH (1956) who demonstrated that thiamine deficient rats were unable to convert tryptophan to formylkynurenine. A partial depletion of apoenzyme in the athiaminotic state has been suggested by a number of investigators. BANGA, OCHOA and PETERS (1939) showed that in extreme depletion, pyruvic oxidase activity is not fully restored by the *in vitro* addition of cocarboxylase. BRIN, SHOHET and DAVIDSON (1958), studying the effect of thiamine deficiency of the glucose oxidative pathway of rat erythrocyte haemolysates, observed incomplete reversal of increased ribose and lactate accumulation, of decreased oxygen consumption and oxidation of [2-^{14}C] glucose to [^{14}C]O_2 when either cocarboxylase or thiamine hydrochloride were added *in vitro*. The *in vivo* administration of vitamin did however restore the glucose oxidative pathway (BRIN *et al.*, 1960). Similar observations were made by BRUNS *et al.* (1958, 1959).

Thus one could postulate that the severe and irreversible histopathological changes which can be observed in some of the avitaminous brains may be the result of enzyme complex dissociation brought about by either a selective deficiency of coenzyme, or apoenzyme or both. The selective vulnerability of certain parts of the brain does not appear to depend on either the total coenzyme content, the rate, or the extent of depletion. Amongst the various biochemical factors which could explain selective

vulnerability, differences in apoenzyme content (specific proteins) and variations in the relative activity of thiamine dependent enzyme systems need to be explored. This latter point is presently under investigation in this laboratory.

SUMMARY

The total thiamine (free and phosphorylated) content of sciatic nerve, spinal cord, and ten subdivisions of the brain of deficient Wistar rats at various stages of depletion and replenishment has been measured by a quantitative histochemical micromethod.

The rate and extent of thiamine depletion were quite uniform. Total thiamine loss ranged from 89 to 76 per cent in the various parts sampled.

Total brain thiamine levels had to be reduced to less than 20 per cent of normal before severe signs and symptoms of deficiency and irreversible histopathological changes became manifest.

There appears to be no correlation between tissue thiamine levels and the susceptibility of certain parts of the neuraxis to vitamin B_1 deprivation.

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RELATIONSHIP OF AMMONIA INTOXICATION TO CONVULSIONS AND COMA IN RATS*

F. NAVAZIO, T. GERRITSEN† and G. J. WRIGHT
L. B. Mendel Research Laboratory, Elgin State Hospital, Elgin, Illinois

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THERE are several reports in the literature demonstrating the relationship between ammonia and hepatic coma (NAJARIAN and HARPER, 1956; MANNIG and DELP, 1958). The possible involvement of ammonia in certain neurological disorders is shown by the increase of free ammonia levels in the brain during experimental convulsions in animals (RICHTER and DAWSON, 1948) and after trauma and/or irritation of the central nervous system (THORN and HEIMANN, 1958). Furthermore, certain drugs known to have anti-convulsive activity (e.g. barbiturates) lower the brain ammonia level (RICHTER and DAWSON, 1948; ANSELL and RICHTER, 1954). In laboratory animals a syndrome with convulsions and coma can be induced by large amounts of ammonium salts (GREENSTEIN *et al.*, 1956; CLARK and EISEMAN, 1958). Arginine and related compounds have been shown to decrease the ammonia level in blood and tissues and to be protective against death following administration of an $LD_{99\cdot9}$ dose of ammonium salts (NAJARIAN and HARPER, 1956; GREENSTEIN *et al.*, 1956).

In this paper data are presented to show the effect of ammonium acetate administration on blood pH and ammonia concentration as well as ammonia levels in brain. The effect of carbon tetrachloride poisoning on ammonia intoxication is demonstrated.

EXPERIMENTAL

Experiments were performed on male albino rats of the Sprague-Dawley strain obtained in two lots, the first lot weighing 300 to 380 g, the second weighing 200 to 300 g. Prior to use, the animals were fasted for 24 hr but allowed access to water. In the first experiment 34 rats were injected intraperitoneally with ammonium acetate equivalent in amount to 7·8 m-moles/kg body weight, dissolved in 3 ml water. In the second experiment 12 rats were injected as before with ammonium acetate equivalent to 4·0 m-moles/kg body weight dissolved in 3 ml water, and killed at predetermined intervals. The animals were injected with Nembutal (5 mg/100 g body weight) from 5 to 15 min before death. In agreement with the observations of RICHTER and DAWSON (1948), Nembutal anaesthesia did not affect the level of ammonia in blood and brain for the observed period of 15 min following the injection. Blood for pH and ammonia determinations was obtained by veni-puncture of the left renal vein. For pH determinations heparin (5 mg/2 ml) was used as an anticoagulant while potassium oxalate (20 mg/3 ml) was used as an anticoagulant for ammonia determinations. Immediately after the blood was drawn, the animal's head and shoulders were placed in a dry ice–acetone mixture for 30 sec, after which the frozen brain was removed and weighed. Brain extracts were prepared by grinding the frozen brain tissue in 8·5 ml of a 0·1 M-phosphate buffer, pH 6·8. The homogenate was centrifuged for 1 min at 500 **g** in the cold. Ammonia was determined in blood and in brain extracts according to the method of CONWAY (1958). Blood pH values were determined with a Beckman blood-pH glass electrode. In continuous infusion experiments, ammonium acetate solution was injected directly into the peritoneal cavity. Twelve animals were injected subcutaneously with carbon tetrachloride in order to produce liver damage (0·1 ml/100 g body wt).

* Supported by a grant-in-aid from the Illinois Mental Health Fund.
† Present address, Department of Pediatrics, University of Wisconsin, Madison 6, Wisconsin.

RESULTS

Basal blood ammonia values ranged from 0·8 to 2 μg NH_3-N/ml of blood. These were slightly higher than the values reported by previous authors (FOLIN and DENIS, 1912; PARNAS and HELLER, 1924; WARREN and NATHAN, 1958). The basal values for brain ammonia ranged from 18 to 24 μg NH_3-N/g (wet weight) in the first series, and 10 to 12 μg/g in the rest of the animals. These data were in good agreement with basal values previously reported in the literature (RICHTER and DAWSON, 1948, found

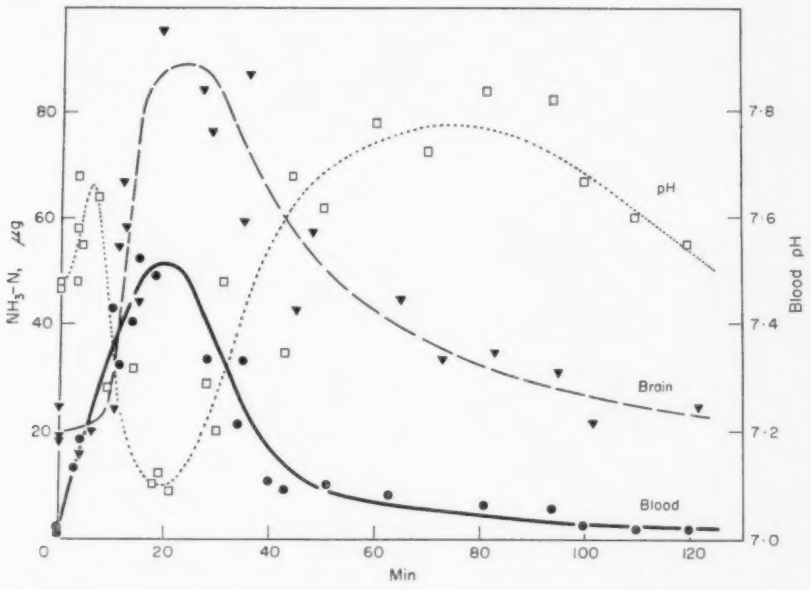

FIG. 1.—Ammonia concentration in the blood and brain, and blood pH, following the injection of ammonium acetate in rats (7·8 m-moles/kg). Blood ammonia values (●) reported in μg/ml blood; brain ammonia values (▼) reported in μg/g (wet weight); blood pH (□).

0·47 mg NH_3/100 g dry weight, which is equivalent to 19·4 μg NH_3-N/g wet weight. Similar values were obtained by THORN and HEIMANN, 1958).

The dose, in the first experiment, was slightly less than the LD_{50} dose reported by GREENSTEIN *et al.* (1956), but it usually produced coma preceded by hypertonus and convulsions. After the injection, the ammonia concentration in the blood increased to 20 times the basal value in 8 to 10 min (Fig. 1). None of the characteristic symptoms was detected before this concentration was attained and no significant increase in brain ammonia was observed. When the blood ammonia concentration increased to more than 20 times its basal value, there was a sudden rise in brain ammonia content, which reached a maximum of approximately 100 μg/g (wet weight) between 10 and 26 min after the injection. When the concentration of ammonia in the brain reached approximately 50 μg NH_3-N/g, contractions and occasional tetanus occurred followed by coma. Although the animals started to recover from the comatose state approximately 70 min after the onset of coma, basal levels of ammonia in blood and brain were not observed until 2 hr after the injection of ammonium acetate. The blood pH

(Fig. 1) rose during the first few minutes and then dropped to pH 7·1 after 18 min, the time of the most severe contractions.

Alkalosis developed subsequently and the pH returned to normal only after 2 hr.

In the second experiment, a maximal concentration of about 20 μg NH_3-N/ml was reached in the blood after 5 to 8 min (Fig. 2). The brain ammonia, which normally in this group of rats was only 10 to 12 μg/g, reached a peak of 20 to 25 μg/g brain after 12 to 16 min but had returned to basal levels within 30 min. No symptoms or signs of coma or convulsions were detected, and the blood pH did not change.

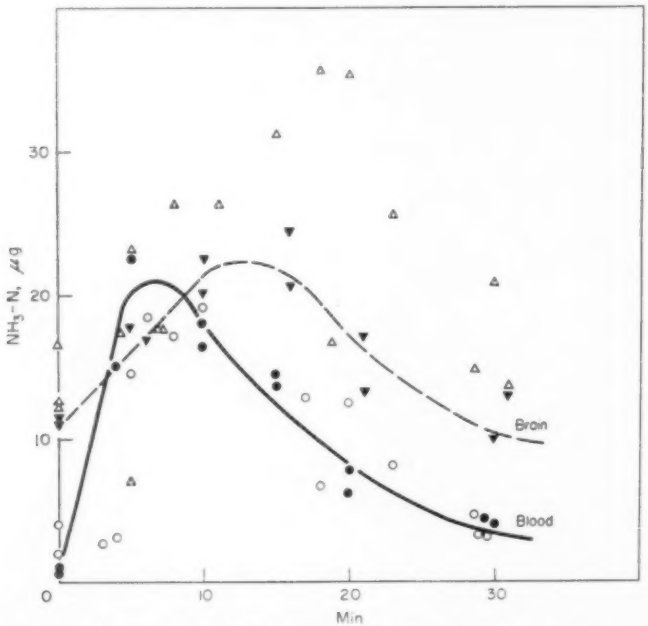

FIG. 2.—Ammonia concentration in blood and brain of healthy rats, and carbon tetrachloride poisoned rats, following the injection of ammonium acetate (4·0 m-moles/kg). Blood values are reported in μg/ml blood: healthy rats (●); carbon tetrachloride poisoned rats (○). Brain values are reported in μg/g (wet weight): healthy rats (▼); carbon tetrachloride poisoned rats (△).

When ammonium acetate (4·0 m-moles/kg body weight) was injected into rats treated 24 hr prior to experiment with a subcutaneous injection of carbon tetrachloride (0·1 ml/100 g body weight), the observed blood ammonia values fell well within the range of the control curve. The zero time values were slightly higher, and an increase of ammonia did not appear in the blood for approximately 3 to 4 min (Fig. 2). The concentration of ammonia in the brain of the carbon tetrachloride treated rats increased to 30–40 μg NH_3-N/g after 15 to 20 min, while all of the rats exhibited signs of hyperreactivity. A similar dose of ammonium acetate in rats which had not been injected with carbon tetrachloride produced no such effect.

The data in Table 1 were obtained from experiments during which 3 rats were continuously infused with ammonium acetate solution (1 m-mole/2 ml/hr) for 6 to 7 hr. Analysis of blood, sampled at regular time intervals, showed that ammonia levels were

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increased from 5 to 20 times the basal value during a period of several hours. Nevertheless, at the end of the experiments, the brain ammonia values were within the basal range.

DISCUSSION

The data presented in this paper demonstrate that in the healthy adult rat, significant increases in brain ammonia levels occurred only when the concentration of ammonia in the blood exceeded 20 times the basal value of 1 μg/ml. BESSMAN and BESSMAN (1955) state that in hepatic coma the brain starts taking up ammonia as soon

TABLE 1.—THE EFFECT OF CONTINUOUS INTRAPERITONEAL INFUSION OF AMMONIUM ACETATE SOLUTION (0·5 M) IN RATS

The average rate of infusion was 2 ml (1 m-mole) per hr.
The animals were killed after 6–7 hr.

Rat No.	μg NH_3-N/ml blood after					μg NH_3-N/g brain after
	0 hr	2 hr	3½ hr	5 hr	6–7 hr	6–7 hr
1. (350 g)	<1*			15·0	17·6	14·9
2. (310 g)	<1	4·7			8·9	13·4
3. (305 g)	<1		5·5		8·0	14·7

* Basal values for blood ammonia in this series of rats were all >1 μg NH_3-N/ml blood.

as the blood level rises above 1 μg/ml, and that the intensity of the neurological symptoms parallels the concentration of ammonia in the arterial blood. In our experiments the initiation of neurological symptoms, such as hyperreactivity, convulsions and coma, occurred only after the brain ammonia levels exceeded 2 or 3 times the basal value. The difference between BESSMAN's findings and the data reported here is that the present results are from fast, short-term experiments, in which large amounts of ammonia diffuse into the blood in the comparatively short period of 5 to 10 min while, in the case of a patient with liver disease, small amounts of ammonia are formed and absorbed over a long period of time. According to BESSMAN (1955, 1956) ammonia taken up in the brain is directly utilized in the chemical reactions: α-keto-glutarate + $NH_3 \rightarrow$ glutamate; glutamate + $NH_3 \rightarrow$ glutamine. A small amount of ammonia can push this reaction to the right (VON EULER *et al.*, 1938). The rapid influx of ammonia could possibly cause rapid exhaustion of the α-keto-glutarate in the brain. As a consequence of the exhaustion of compounds taking part in the Krebs cycle, decreases in both oxygen utilization and high energy phosphate production may result. Such alteration could be related to the onset of hepatic coma. Since the concentration of brain ammonia is regulated in the healthy animal by the so-called 'blood-brain barrier', as well as the various rates of brain metabolic and detoxicating mechanisms, ammonia can start to increase in the brain only after the blood ammonia level has altered the functioning of the regulatory mechanisms and exceeded the capacity of the normal metabolic pathways to detoxicate it.

The pH curve as shown in Fig. 1 is apparently a product of several processes. The fall in blood pH is accompanied by a rise in blood ammonia, followed by a rise in blood pH from 7·1 to 7·6 which correlates with a decrease in blood and brain ammonia levels. Seen as such, these facts are in contradiction with the hypothesis of WARREN

et al. (1958) who observed that an artificial increase of blood pH facilitates the influx of ammonia into the tissues and vice versa. It is, however, questionable whether small amounts of NH_4^+ ions, diffused into the bloodstream from the intraperitoneal cavity could have a measurable influence on the blood pH, while it is obvious that the large amounts of lactic acid and pyruvic acid formed during the convulsions appreciably lowered the pH. These data agree with the report of LAWRENCE *et al.* (1957), whose Eck fistula dogs showed a lower blood pH and a rise in total blood ammonia levels. BRODIE and WOODBURY (1957) estimated normal rat cerebral cortical intracellular pH to be $7{\cdot}04 \pm 0{\cdot}07$. During respiratory acidosis, caused by inhalation of CO_2, the plasma pH decreased to $6{\cdot}95 \pm 0{\cdot}06$. As the greater concentration of ammonia should be on the side of the membrane where the pH is lower, the fact that the cerebral pH always tended to stay below the blood pH may explain why a blood ammonia level of 50 μg/ml was obtained when the brain level went up to 90 μg/g.

In animals poisoned with carbon tetrachloride, a different picture is seen. Blood ammonia levels which caused a slight rise in the ammonia level of the brain (Fig. 2), and no neurological symptoms in normal rats, produced significantly higher brain ammonia values and signs of hyperreactivity in animals with liver damage. This observation makes it probable that, in cases of liver damage, the increased ammonia level in the blood is not the only direct cause of the increased ammonia level in the brain; and, therefore, of the neurological symptoms. The absence or decreased concentration of an essential liver factor (compare GEIGER *et al.*, 1954, 1956) might diminish the capacity of the brain to cope with an influx of ammonia. This diminished capacity could be due to a partial break-down of the regulatory mechanisms of the 'blood-brain barrier', a decreased activity of the glutamine producing enzyme system, or both. Conversely, another interpretation may be placed on the data in the light of more recent evidence (BRODY, 1959; CALVERT and BRODY, 1960; MOORE and BRODY, 1960; BRODY and CALVERT, 1960) which indicates that the primary effect of carbon tetrachloride poisoning is exerted on the sympathetic nervous system and secondarily causes a massive liberation of catecholamines from the adrenal glands resulting in anoxia and causing the observed changes in liver mitochondria and liver-fat aberrations. It is entirely possible that the change in adrenal function as well as the stimulation of the sympathetic nervous system could also result in alteration of the regulatory mechanisms of the 'blood-brain barrier'. Either of the above mechanisms could explain the neuropsychiatric symptoms observed in human patients with liver disease and increased concentrations of ammonia in the blood even though these levels are comparatively low when related to the amount necessary to cause difficulty due to ammonia alone.

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SUMMARY

Significant increases in brain ammonia levels in the rat occurred only at a blood ammonia level of 20 times (20 μg/ml) the basal value or higher. Neurological symptoms occurred at a brain ammonia level of 40 μg/g or more.

Blood ammonia values which caused a slight rise in the ammonia level of normal rat brain, but no neurological symptoms, produced significantly higher brain ammonia values and signs of hyperreactivity in rats poisoned with carbon tetrachloride.

Acknowledgements—Our thanks are due to Dr. M. K. HORWITT for his interest and support of this work, and to Miss KATHRYN RUPP for her technical assistance.

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γ-AMINOBUTYRIC ACID AND ITS ACTION ON THE ADRENAL CORTEX—II. EFFECTS OF γ-AMINOBUTYRIC ACID ON THE EXCRETION OF 17-HYDROXYCORTICOSTEROIDS IN URINE

MUTSUTOSHI KOSAKA and AKITANE MORI
Departments of Neurology and Psychiatry and of Neurosurgery, Okayama University Medical School, Okayama, Japan

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IN A previous report (MORI and KOSAKA, 1961) it was found that γ-aminobutyric acid (GABA)* brought about a decrease in the ascorbic acid content of the adrenals through the action of the hypophysis. According to NELSON (1960) the injection of adrenocorticotrophic hormone (ACTH) induces a decrease in adrenal ascorbic acid and a rapid increase in corticosterone in the adrenal vein. From our results in the previous report it appeared that the action of GABA was similar to that of ACTH. The actions of GABA, ACTH and pantothenic acid have now been investigated further by measuring the amount of 17-hydroxycorticosteroids (17-OH-CS) in urine.

MATERIALS AND METHODS

A catheter was placed in the ureter of untreated female rabbits and control urine was collected. Then GABA, ACTH, or pantothenic acid was injected into the auricular vein. Urine was collected at intervals of 1, 2, 3, 4, and 5 hr after the injection. The 17-OH-CS content of each sample was measured, using the methods of SILBER and PORTER (1954) and BONGIOVANNI (1954). To prevent function of the hypophysis we employed electrical cauterization as described in our previous experiments (marked as 'hypophysectomized' in Figs. MORI and KOSAKA, 1961).

RESULTS

The concentrations of free and conjugated 17-OH-CS in rabbit urine were approximately the same, ranging from 6 to 8 μg/ml (Table 1). In these experiments we used 7,500 Fishman units of β-glucuronidase† for 2 ml of urine and incubated the samples for 20 hr at 38°, pH 6·5. As the free and conjugated steroids were about equal in amount, only the free type of 17-OH-CS and its fluctuations were determined subsequently.

The injection of ACTH (2 i.u./kg) into normal rabbits increased the excretion of corticoids only slightly (Fig. 1, graph *a*). It appears that when the hypophysis is intact, this amount of exogenous ACTH has only slight effect. On the other hand, there was a marked increase in corticoid excretion after injection of 2 i.u. of ACTH per kg when the hypophysis was cauterized; in this case the urinary corticoid level was extremely low before injection (Fig. 1, graph *b*).

Changes in 17-OH-CS after the injection of 200 mg of GABA per kg (Fig. 2) show that excretion of 17-OH-CS in the urine of normal rabbits is doubled within an hour of

* The abbreviations used are: GABA, γ-aminobutyric acid; 17-OH-CS, 17-hydroxycorticosteroids; ACTH, adrenocorticotrophic hormone.

† β-glucuronidase (Ketodase): A product of Warner-Chilcott, Laboratory Supply Division, Morris Plains, N.J. containing 5,000 Fishman units in each ml.

GABA injection (Fig. 2, graph *a*). On the other hand, after cauterization of the hypophysis, rabbits that had been injected with the same amount of GABA showed no rise in the corticoid output (Fig. 2, graph *b*). These results indicate that GABA accelerates corticoid excretion by way of the hypophysis.

TABLE 1.—EXCRETION OF URINARY 17-OH-CS

Rabbit No.	Time (hr)	17-OH-CS (μg/ml)	
		Free	Conjugated
III	1	7·5	6·6
	2	7·1	7·3
	3	7·1	7·9
	4	7·3	7·3
IV	1	5·9	5·9
	2	6·1	6·1
	3	5·9	6·7

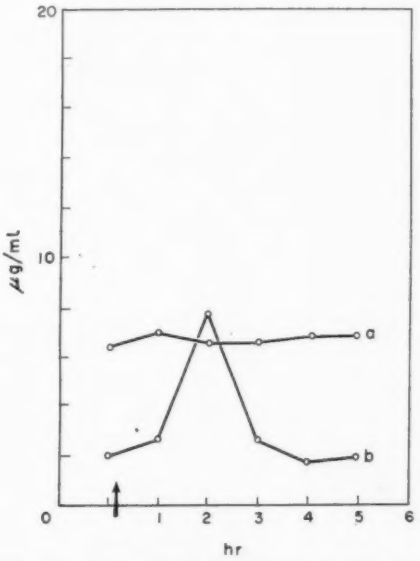

FIG. 1.—Changes in 17-OH-CS excreted in the urine after the injection of 2·0 i.u. ACTH/kg
a: mean of 2 cases of normal rabbits
b: mean of 3 cases of hypophysectomized rabbits

Changes in the urinary 17-OH-CS values after the injection of 20 mg of pantothenic acid per kg body weight are shown in Fig. 3. KOSAKA (1960) observed an increase in the conjugated 17-OH-CS content of the urine after oral administration of pantothenic acid to schizophrenic patients. The unconjugated steroids in normal rabbit urine (Fig. 3, graph *a*) did not change much after injection of pantothenic acid, nor was there any change in rabbits with cauterized hypophyses (Fig. 3, graph *b*).

Homopantothenic acid, the substance in which the β-alanine component of

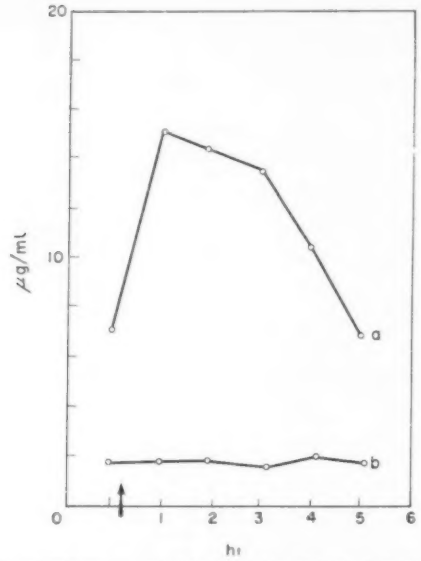

FIG. 2.—Changes in 17-OH-CS excreted in the urine after the injection of 200 mg GABA/kg
a: mean of 4 cases of normal rabbits
b: mean of 2 cases of hypophysectomized rabbits

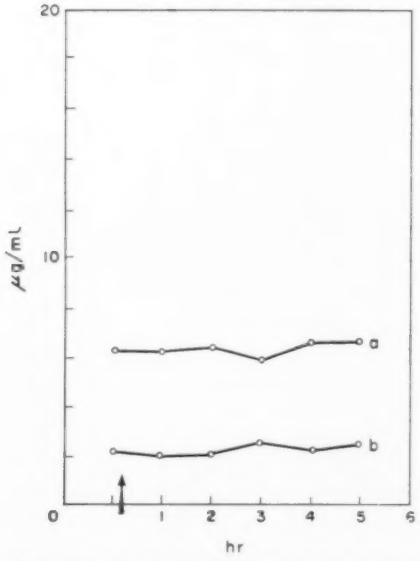

FIG. 3.—Changes in 17-OH-CS excreted in the urine after the injection of 20 mg pantothenic acid/kg
a: mean of 3 cases of normal rabbits
b: mean of 2 cases of hypophysectomized rabbits

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pantothenic acid is replaced by γ-aminobutyric acid, was injected at a rate of 20 mg per kg body weight. It had no effect on the 17-OH-CS content of the normal rabbit urine (Fig. 4, graph *a*) but in animals with cauterized hypophyses it appeared to cause a slight increase in 17-OH-CS excretion.

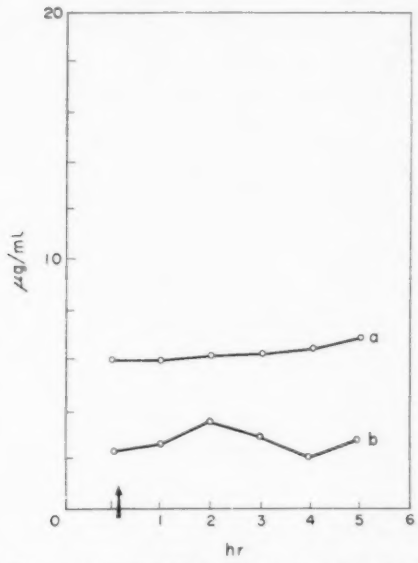

FIG. 4.—Changes in 17-OH-CS excreted in the urine after the injection of 20 mg homopantothenic acid/kg
a: mean of 3 cases of normal rabbits
b: mean of 3 cases of hypophysectomized rabbits

DISCUSSION

GABA stimulates the adrenal cortex through the hypophysis, presumably by release of ACTH, and enhances the 17-OH-CS excretion in the urine, showing the maximal values within one hour after its administration. The decrease in adrenal ascorbic acid after GABA administration reached its maximum 10 min after injection as described in our previous report (MORI and KOSAKA, 1961). According to NELSON (1960), a rapid increase of ascorbic acid is observed immediately after the injection of ACTH and 4 min later a slow decrease occurs; coincident with these fluctuations, corticosterone in the adrenal vein increases rapidly. From these results, it appears that the increase in 17-OH-CS excreted in the urine likewise reaches its maximum within 10 min after GABA injection. Where the role of the hypophysis has been eliminated, 17-OH-CS excretion in the urine is not increased by GABA. In intact animals the injected GABA appears to enhance the secretion of ACTH but whether this or other factors are involved in the increase of 17-OH-CS excreted in the urine, is not yet known. Homopantothenic acid acts somewhat like ACTH, although with less marked effect; in rabbits with cauterized hypophyses it stimulates the adrenal cortex and increases the excretion of 17-OH-CS. GABA differs from homopantothenic acid in this respect, although the latter has pantoic acid and GABA in its structure. The relationships of GABA, pantothenic acid, and homopantothenic acid

in connection with the function of the hypophysis and adrenal cortex will be studied further.

SUMMARY

We have investigated the stimulative action of GABA on the adrenal cortex of rabbits. It appears to stimulate through the hypophysis resulting in increased excretion of 17-OH-CS in the urine. GABA seems to accelerate the secretion of ACTH.

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PRELIMINARY NOTE

Relationship between mesencephalic–hypothalamic concentration of 5–hydroxytryptamine and cortical electrical activity of cats*

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INTRACAROTIC injection of small doses of 5-hydroxytryptophan (5-HTP) in the 'midpontine-pretrigeminal' preparation of the cat gives rise to electroencephalographic (EEG) synchronization (GLÄSSER and MANTEGAZZINI, 1960); transection of the midbrain produces a similar but more pronounced synchronization. In the experiments reported here an attempt was made to establish whether midbrain transection and 5-HTP injection, which have similar EEG effects, cause similar variations in the

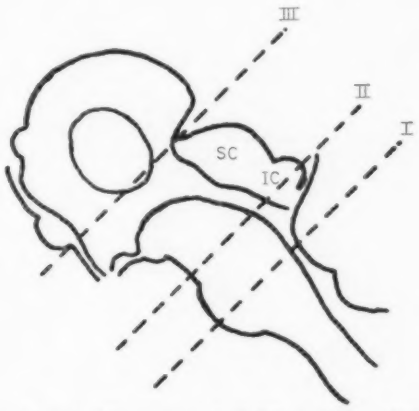

FIG. 1—Midpontine-pretrigeminal(I), mesencephalic(II), and rostral(III), transections of cat brain.
I.C.: Inferior colliculi S.C.: Superior colliculi

concentration of 5-hydroxytryptamine (5-HT) in the mesencephalon and hypothalamus and on this basis whether there is any difference in the 5-HT concentration in the mesencephalon and hypothalamus during periods of sleep and wakefulness as revealed by EEG and ocular patterns.

In all experiments a transection was made at the midpontine level just in front of the trigeminal rootlets under ether anaesthesia (Section I, Fig. 1). EEG and ocular patterns of this preparation (midpontine-pretrigeminal preparation) were considered to be typical of the waking state (BATINI, MORUZZI, PALESTINI, ROSSI and ZANCHETTI, 1959). In some experiments a second section was made at the mesencephalic level through the posterior half of the inferior colliculi reaching the basilar bone 1-2 mm in front of the superior border of the pons (Section II, Fig. 1). After transection at this level EEG and ocular patterns were typical of the sleeping state. The DL-5-HTP was injected slowly (1-2 mg/min) into the right carotid artery, as described by GLÄSSER and MANTEGAZZINI (1960). Animals were killed by decapitation. The theca and tentorium were removed and a third transection was made between the anterior border of the superior colliculi and the posterior border of the optic chiasma (Section III, Fig. 1). That part of the brain contained between the second and third transections (mesencephalon and hypothalamus minus hypophysis) was removed and frozen at $-70°$, 5–7 min after decapitation.

* Preliminary note.

All transections were made with a spatula oriented stereotaxically at 45°. The 5-HT was extracted and estimated according to the method of ERSPAMER (1940). In some experiments the 5-HT concentration of the two symmetrical halves of the mesencephalon–hypothalamus was determined.

The following results were obtained. In the midpontine pretrigeminal preparations which were characterized by EEG and ocular patterns considered to be typical of the waking state, the mesencephalic-hypothalamic concentration of 5-HT was 0·50 ± 0·08 μg/g fresh tissue (5 animals). Injection of 5-HTP (5 to 10 mg) into the right carotid artery gave rise to EEG synchronization of the right hemisphere (GLÄSSER and MANTEGAZZINI, 1960). This EEG pattern appeared 6–11 min after injection was started and was strictly unilateral. Visual or olfactory stimuli produced an 'arousal reaction'. Animals were killed 4–6 min after the appearance of the EEG effect. The mesencephalic-hypothalamic concentration of 5-HT of the right and left side were 0·85 ± 0·10 μg/g and 0·57 ±0·08 μg/g respectively, showing a significant difference of about 50 per cent (8 animals; $P < 0·01$). In controls in which 5-HTP had not been injected the difference in 5-HT concentration of the two halves was about 4–5 per cent (5 animals). Mesencephalic transection (Section II, Fig. 1) induced EEG and ocular patterns which are considered to be typical of the sleeping state. The effect on EEG was greater than that induced by 5-HTP injection. Olfactory stimuli rarely brought out even a brief 'arousal reaction', while visual stimuli were always without effect. The 5-HT concentration of the mesencephalic-hypothalamic area was 0·55 ± 0·19 μg/g (6 animals).

Summing up, the 5-HT concentration of the mesencephalic-hypothalamic area in preparations representing a state of sleep (mesencephalic transection) does not differ significantly ($P > 0·5$) from that of preparations representing a state of wakefulness (midpontine-pretrigeminal transection). The 5-HT concentration of the mesencephalic-hypothalamic area in the state of encephalographic synchronization induced by 5-HTP is higher than it is in the more pronounced EEG synchronization and ocular sleep brought about by midbrain transection.

These findings suggest that there is no relation between the 5-HT concentration of the mesencephalon-hypothalamus and the sleep–wakefulness state as defined. This, of course, does not mean that 5-HT might not interfere with the activity of the structures considered above.

Institute of Pharmacology
University of Parma
Farmitalia Research Laboratories
Milan, Italy.

A. R. ANTONELLI*
G. BERTACCINI
P. MANTEGAZZINI

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* Present address: Clinica Otorinolaringoiatrica dell'Università-Sassari, Italy.

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ANNOUNCEMENT

World Federation of Neurology

Commission of Neurochemistry International Symposium on
Enzymic Activity in the Central Nervous System

AN International Symposium will take place in Göteborg, Sweden, on June 17th–21st, 1962, under the auspices of the Neurochemical Commission of the World Federation of Neurology. The subject will be the enzymic activity of the central nervous system, and will include the following topics:

1. Enzymic mechanisms in amino acid and protein metabolism.
2. Enzymic mechanisms in lipid metabolism.
3. Enzymic activity in relation to function and to pathological conditions.

This meeting is at present open to all interested in the field, but for administrative reasons the number attending will be limited. Emphasis will be placed on short, original communications, and it is hoped that there will be ample opportunity for exchange of views between workers in the clinical fields and those in the basic sciences. The offical languages will be English, French and German. The registration fee will be Sw. Cr. 70 ($15 or £5.5.0), payable on registration; the closing date for this is March 1st, 1962.

Those interested in attending should communicate with one of the secretaries, from whom further information can be obtained. Those wishing to present a paper will have to submit an abstract in English, of not more than 400 words, to one of the secretaries before December 31st, 1961. All abstracts should be accompanied by a registration form. The Organizing Committee will select the papers to be included in the programme and they reserve the right to have any paper summarized by a suitable reporter or read by title only.

Secretaries

A. LOWENTHAL
Institute Bunge
59 rue Philippe Williot
Berchem-Snvers
Belgium

G. B. ANSELL,
Department of
Experimental Psychiatry
Medical School
Birmingham 15
England.

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Papers to be published in future issues

F. WOLFGRAM and A. S. ROSE: A study of some component proteins of central and peripheral nerve myelin

D. H. CLOUET and H. WAELSCH: Amino acid and protein metabolism of the brain—VII

D. H. CLOUET and H. WAELSCH: Amino acid and protein metabolism of the brain—VIII

E. KOENIG and G. B. KOELLE: Mode of regeneration of acetylcholinesterase in cholinergic neurons following irreversible inactivation

A. LAJTHA and J. TOTH: The brain barrier system—II

M. D. MORRIS and I. L. CHAIKOFF: Concerning incorporation of labelled cholesterol, fed to the mothers, into brain cholesterol of 20-day-old suckling rats

R. BLOMSTRAND and F. NAKAYAMA: Neutron activation paper chromatographic analysis of human cerebrospinal fluid

S. LØVTRUP: The subcellular localization of glutamic decarboxylase in rat brain

M. K. GAITONDE: The composition of brain lipoprotein fractions obtained by different methods

M. B. LEES, L. AMADUCCI and B. H. WAKSMAN: Further studies of the properties of the encephalitogenic component(s) of bovine nervous tissue

S. BALAKRISHNAN, H. GOODWIN and J. N. CUMINGS: The distribution of phosphorus-containing lipid compounds in the human brain

H. H. HILLMAN: Membrane potentials in guinea-pig cerebral cortex slices in vitro. Their dependence on substrate and oxygen. The effect of clupein and of ganglioside preparations

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A STUDY OF SOME COMPONENT PROTEINS OF CENTRAL AND PERIPHERAL NERVE MYELIN*

FREDERICK WOLFGRAM and AUGUSTUS S. ROSE
Division of Neurology, Department of Medicine, University of California at Los Angeles and the Los Angeles Veterans Administration Center

(*Received* 10 *February* 1961)

HIGHLY specific and sensitive methods have recently been introduced for the histochemical localization of indole derivatives in tissue sections (GLENNER and LILLIE, 1957; GLENNER, 1957; ADAMS, 1957). These techniques are particularly valuable for the visualization of tryptophan in proteins. It was noted by the above authors that peripheral nerve 'neurokeratin' was strongly reactive while the axons were negative. Although it was assumed that the reacting protein was neurokeratin, *i.e.*, the trypsin resistant protein of myelin, no experimental evidence was presented to support such a conclusion. The present investigation began as a study of this tryptophan-containing protein of myelin. In the course of the work, new data were accumulated on the elemental and amino acid compositions of central and peripheral nerve proteolipid proteins. In addition, a method was devised for the isolation of peripheral nerve neurokeratin, this being a fraction that had not previously been isolated.

EXPERIMENTAL

Histochemical methods. The post-coupled *p*-dimethylaminobenzylidene reaction for the visualization of protein bound tryptophan was used as described by GLENNER and LILLIE (1957). Intradural spinal roots and/or sciatic nerves of the frog, chicken, rat, guinea pig, rabbit, cat, dog, sheep, horse, cow and human have been examined by this technique. The blue reaction product of the protein bound tryptophan was identical in intensity and location in all of these species. Spinal cords from most of the above species were also examined. For routine studies paraffin sections were prepared in the usual manner after 3–6 hr fixation in neutral 10% formalin. Longer fixation diminished or abolished the reaction. For studies on the solubility of the protein in organic solvent mixtures, fresh frozen sections were cut in a cryostat at 16 μ, mounted on uncoated slides, and vacuum dried at room temperature. The effects of digestion with trypsin were studied on unfixed frozen sections and on paraffin sections that had been fixed in 80% alcohol and mounted on uncoated slides. Digestion with trypsin was carried out for 2–24 hr in 0·2% trypsin solution (twice crystallized salt-free trypsin, Nutritional Biochemicals Corp.) in 0·1 M-phosphate buffer, pH 7·8. After the solvent extractions or digestion with trypsin the unfixed frozen sections were washed and fixed in neutral 10% formalin for 2–3 hr before proceeding with the histochemical procedure.

Protein isolation methods. Centrum ovale white matter and intradural spinal roots were the tissue sources used for the isolation of the protein fractions of bovine and human origin. Spinal roots were chosen rather than sciatic nerve because the proportion of connective tissue to nerve fibres is lowest at this point in the peripheral nervous system. The proteolipid proteins of both CNS white matter and peripheral nerve were isolated by a modification of the method that FOLCH and LEES (1951) used for the isolation of CNS white matter proteolipid protein. The tissues were weighed and homogenized twice at 4° in a commercial size Waring blendor with 20 vol per gram of chloroform–methanol (2:1). The homogenates were filtered on coarse porosity sintered glass filters and the insoluble residue was dried at 50° and weighed. This residue accounted for about 10 per cent of the wet tissue weight and

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was used for the preparation of the neurokeratin fraction. The chloroform–methanol soluble material was refiltered through Whatman No. 1 paper and taken to dryness *in vacuo* in a rotary evaporator. It was resuspended in a small amount of water and redissolved as much as possible by adding chloroform and then methanol until one phase resulted. This was taken to dryness, and the process repeated six times. At this point the crude proteolipid protein was separated by centrifugation of the chloroform–methanol–water solution. The proteolipid protein was washed twice with water-saturated chloroform–methanol (2:1), once with methanol, and twice with water. It was again washed with methanol and suspended for 18 hr in 50 vol of chloroform–methanol–HCl (200:100:1) to remove any phosphatido-peptides that might be present (FOLCH, 1952; FOLCH and LEBARON, 1953). After centrifugation the proteolipid protein was washed with more of the acidified solvent, then with methanol, and dried. The tissue residue that was originally insoluble in chloroform–methanol was homogenized in 10 vol of 0·1 M-phosphate buffer, pH 7·8, and an equal volume of the buffer containing 0·4% trypsin was added. After layering with toluene the solution was incubated at 37° for one week. The insoluble residue was recovered by centrifugation, washed twice with water, and ground to a fine suspension in an all glass tissue homogenizer. The suspension was incubated at 37° under toluene for 2 days in a 0·1% solution of hyaluronidase in 0·1 M-acetate buffer, pH 5·0, to remove the axonal mucopolysaccarides (ABOOD and ABUL-HAJ, 1956). Preliminary studies had shown that our neurokeratin preparations were contaminated with mucopolysaccharides when hyaluronidase digestion was not used. Again after centrifugation and washing, the residue was incubated for two weeks in two changes of 0·1% collagenase in 0·1 M-tris buffer, pH 7·4, containing mM-$CaCl_2$ (GALLOP, SEIFTER and MEILMAN, 1957). A final digestion in 0·2% trypsin for one week completed the process.

Previous attempts to prepare sciatic nerve neurokeratin failed because peripheral nerve collagen is resistant to digestion with pepsin (FOLCH, LEES and CARR, 1958). The resistance of collagen to digestion with trypsin is well known. Accordingly we digested the collagen of the intradural spinal root homogenates with a commercial collagenase preparation (Nutritional Biochemicals Corporation) derived from *Clostridium histolyticum* (DEBELLIS, MANDL, MACLENNAN and HOWES, 1954). The resultant protein fraction accounted for approximately 0·6 per cent of the wet tissue weight of bovine roots and was judged to be virtually free of collagen by its hydroxyproline content (0·3%). Further studies on the composition of this neurokeratin preparation will be reported in a later communication. It will be considered here only for its tryptophan content and its contribution to the total myelin protein of peripheral nerve. In addition to isolating the above mentioned proteins, a neurosclerin fraction was prepared from bovine CNS white matter by the method of UZMAN (1958). Analyses for nitrogen, phosphorus, and sulphur were performed by Dr. ADALBERT ELECK of Eleck Microanalytical Laboratories, Los Angeles.

Amino acid analyses. The proteins were hydrolysed in evacuated tubes in 6 N-HCl at 110° for 22 hr. Quantitative amino acid analyses of the acid hydrolysates were performed by Miss RUTHE TYSON of Oxford Laboratories, Redwood City, California, according to the method of SPACKMAN, STEIN and MOORE (1958) using ion-exchange chromatography on sulphonated polystyrene resins in a Spinco Model 120 Amino Acid Analyzer. The analytical method is accurate to 100 ± 3 per cent for loads of individual amino acids from 0·1 to 3·0 μmoles. As tryptophan is largely destroyed by acid hydrolysis it was determined separately by hydrolysis in 3·5 N-$Ba(OH)_2$ for 10 hr at 100°. After removal of the barium and adjustment of the pH to 2·5, part was chromatographed on Amberlite IRC-120 at 50° with elution at pH 5·28. The tryptophan was determined spectrophotometrically with ninhydrin.

RESULTS

Histochemistry of tryptophan in myelin proteins

Peripheral nerve. The observations of GLENNER and LILLIE (1957) and ADAMS (1957) on the presence of protein bound tryptophan in myelin and its absence in axons are confirmed (Fig. 1). The properties of this fraction are summarized in Table 1. Extraction of this protein from unfixed frozen sections of peripheral nerve with water saturated chloroform–methanol, as judged by microscopic observation, varied with about 60 to 100 per cent of the material being soluble. Because of this solubility it can be classified as a proteolipid. This protein is insoluble when whole spinal roots are

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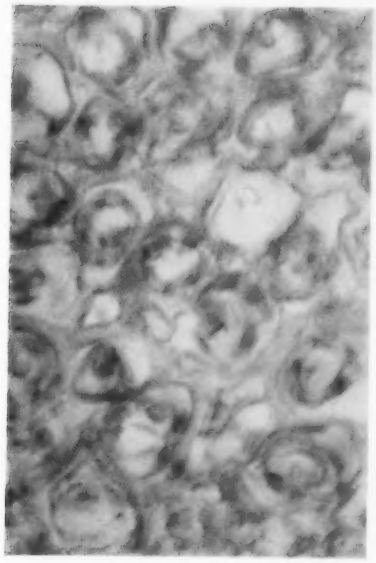

FIG. 1.—Tryptophan (blue colour) in a cross section of human sciatic nerve myelin. Didymium filter. ×1250.

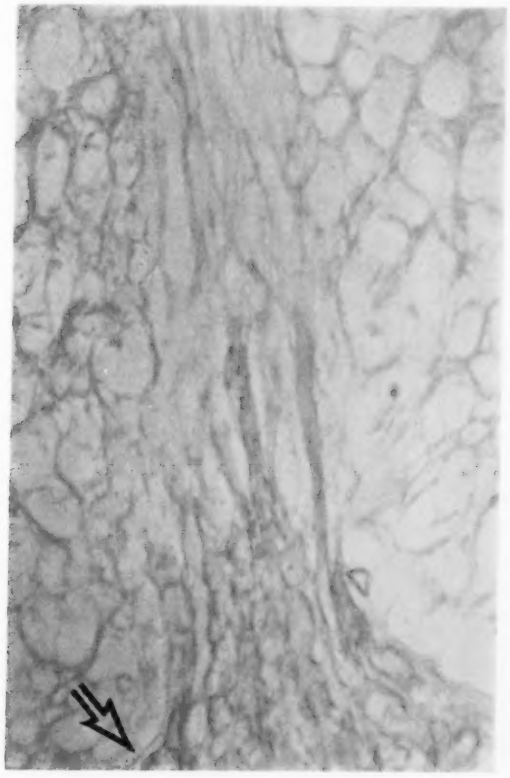

FIG. 2.—Glial-Schwann cell junction of bovine posterior spinal root. The peripheral myelin is the darker blue segment. Arrow indicates pia mater. ×500.

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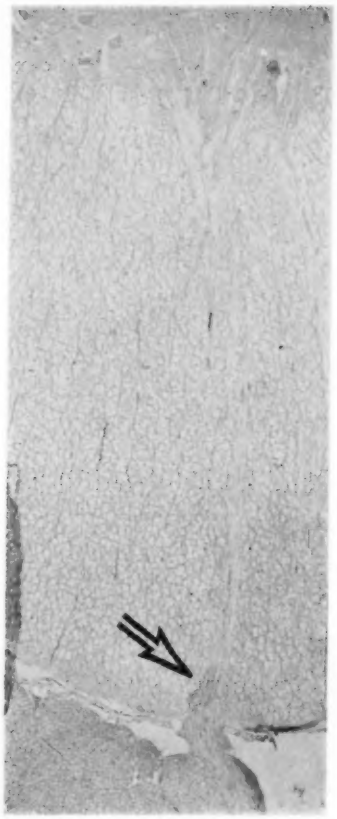

FIG. 3.—Glial-Schwann cell junction and anterior horn of dog spinal cord. Arrow indicates the extent of indentation of peripheral myelin into the cord. ×125.

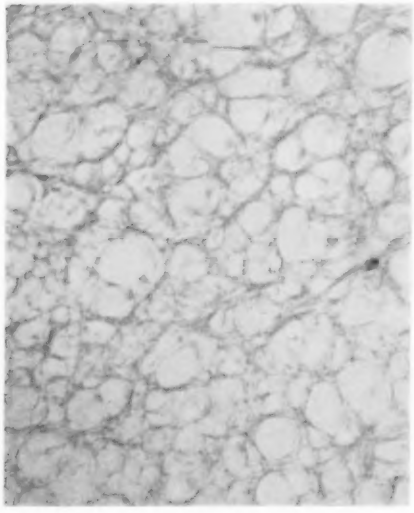

FIG. 4*a*.—Cross-section of bovine spinal cord white matter showing tryptophan in myelin proteolipid protein. ×300.

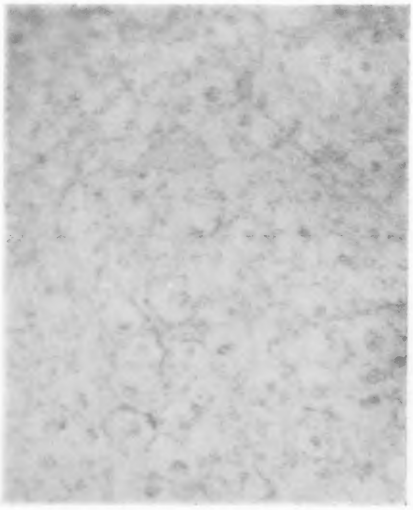

FIG. 4*b*.—Same as Fig. 4*a*, except that the tissue block was soaked in chloroform–methanol (2:1) before fixation. ×300.

soaked in chloroform–methanol. From this, it is inferred that frozen sections constitute an extremely favourable situation for extraction of the protein with the solvent mixture and it is not possible to estimate from the histochemical study alone to what extent the protein would be solubilized when spinal roots are homogenized in chloroform–methanol.

The protein can be extracted from the peripheral myelin equally well by using dry mounted sections in water saturated chloroform–methanol or wet sections in anhydrous chloroform–methanol. It is insoluble when dry sections are extracted with anhydrous chloroform–methanol. A considerable number of other water saturated organic solvent mixtures were tested, among them: toluene–methanol (2:1); chloroform–pyridine; ether–methanol; chloroform–dioxane; and *iso*amylacetate–methanol. The protein was insoluble in all of them.

TABLE 1.—PROPERTIES OF THE TRYPTOPHAN CONTAINING PROTEINS OF CENTRAL AND PERIPHERAL MYELIN THAT ARE VISUALIZED BY THE POST-COUPLED *p*-DIMETHYLAMINOBENZYLIDENE REACTION

	Central myelin (%)	Peripheral myelin (%)
Solubility in anhydrous chloroform–methanol	0	0
Solubility in water saturated chloroform–methanol (2:1)	100	60–100
Solubility in water saturated chloroform–methanol (2:1) after formalin fixation	0	0
Digestion by trypsin (24 hr)	0–20	0

The tryptophan containing protein of peripheral nerve myelin is completely resistant to digestion with trypsin as judged by histological studies on unfixed frozen sections. FOLCH and LEES (1951) have shown that bovine CNS white matter proteolipid protein is resistant to digestion with trypsin.

Central nervous system white matter. All of the histochemically detectable protein bound tryptophan of CNS white matter is in the myelin but the histochemical reaction is much less intense in central fibres than in the periphery. This is particularly noticeable at the glia-Schwann cell junctions in the anterior and posterior spinal roots (Figs. 2 and 3). The less intense reaction in CNS myelin is probably due in part to the thinner myelination of central fibres (see Discussion) but spinal root proteolipid protein also contains more tryptophan than its central analogue.

In contrast to the insolubility of the peripheral nerve protein when spinal roots are soaked in chloroform–methanol, the CNS white matter protein is readily and completely soluble in chloroform–methanol when small blocks of tissue are soaked in the solvent mixture (Figs. 4*a*, 4*b*). This observation is reinforced by the fact that CNS white matter neurokeratin, unlike peripheral nerve neurokeratin, forms a fraction that is relatively insignificant in amount when compared to the proteolipid protein.

Elemental and amino acid compositions of proteolipids

The nitrogen recovered as amino acid nitrogen plus ammonia expressed as percentage of the total nitrogen was 84·6 per cent for human CNS white matter proteolipid protein, 72·3 per cent for human spinal root proteolipid protein, 93·5 per cent

for bovine CNS white matter proteolipid protein,and 91·5 per cent for bovine spinal root proteolipid protein (Table 2). The total sulphur was recovered as amino acid sulphur except for the human CNS white matter proteolipid protein (86 per cent recovery). Ideally, second analyses of 70 hr hydrolysates should be done as some peptide linkages are known to be rather resistant to acid hydrolysis and require longer than 22 hr to be ruptured, and also because some amino acids, particularly the hydroxyl-amino acids, decompose considerably during a 22 hr hydrolysis (HIRS, STEIN and MOORE, 1954). These proteins also contained appreciable amounts of ethanolamine (and possibly other nitrogenous compounds) which could not be accurately measured

TABLE 2.—YIELDS AND ELEMENTAL ANALYSES OF PROTEOLIPID PROTEIN PREPARATIONS

		CNS white matter proteolipid protein	Spinal root proteolipid protein
Human	Yield (% of wet tissue wt)	2·0	0·177
	Nitrogen (% of proteolipid protein)	13·71	14·21
	Phosphorus (% of proteolipid protein)	0·13	0·80
	Sulphur (% of proteolipid protein)	1·65	0·78
Bovine	Yield (% of wet tissue wt)	2·1	0·103
	Nitrogen (% of proteolipid protein)	13·40	15·17
	Phosphorus (% of proteolipid protein)	0·10	0·08
	Sulphur (% of proteolipid protein)	1·60	1·35

by the method as used here, and this contributed to the discrepancy between the recovered nitrogen and the total nitrogen. In this regard it was often observed that the higher the phosphorus content of the proteolipid sample the lower the recovery of total nitrogen as amino acid nitrogen. This is presumably due to the contamination of the protein with phosphatides.

Two interesting features of the proteolipid amino acid analyses (Table 3) are: (1) the relative constancy of the amino acid pattern regardless of whether the proteolipids are human or bovine, of central or peripheral origin, and (2) in all of the proteolipids analysed glycine, alanine, leucine, and *iso*leucine comprised more than 35 per cent of the eighteen constituent amino acids. The amino acid composition of bovine CNS white matter proteolipid protein as determined in the present investigation has been compared (Table 4) with the analysis given by FOLCH and LEES (1951) and the results are in close agreement except for methionine, cystine and serine. More recently, FOLCH (1959) reported on a definitive study of the lipid and amino acid composition of bovine CNS white matter proteolipids. The amino acids composition of our preparations are also in good agreement with these results. It is interesting that FOLCH observed that proteolipids in solution have a strong absorption at 280 mμ which he correctly ascribed to their rather high tryptophan content.

Neurosclerin. UZMAN and ROSEN (1958) noted that their specimens of neurosclerin from whole mouse or monkey brain contained 45–49% glycine, alanine, leucine, and *iso*leucine. Since they postulated that neurosclerin is a myelin protein we prepared a single sample from bovine CNS white matter. The yield was 1·8 per cent of the wet tissue weight and the elemental analysis was 13·55% nitrogen, 0·48% phosphorus, and 1·91% sulphur. The yield of this sample from white matter was larger than that

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TABLE 3.—AMINO ACID COMPOSITION OF PROTEOLIPID PROTEINS

Amino acid	Human CNS white matter		Human spinal root		Bovine CNS white matter		Bovine spinal root	
	μmole/mg	% total	μmole/mg	% total	μmole/mg	% total	μmole/mg	% total
Aspartic acid	0·281	4·04	0·410	6·03	0·278	3·67	0·417	4·84
Glutamic acid	0·399	5·74	0·334	4·91	0·420	5·55	0·471	5·46
Glycine	0·721	10·38	0·424	6·24	0·760	10·05	0·700	8·12
Alanine	0·769	11·07	0·704	10·36	0·870	11·51	0·864	10·03
Leucine	0·750	10·80	0·857	12·61	0·783	10·36	1·023	11·88
*iso*Leucine	0·321	4·62	0·250	3·67	0·352	4·65	0·456	5·29
Serine	0·298	4·29	0·421	6·19	0·339	4·48	0·633	7·35
Threonine	0·512	7·37	0·365	5·37	0·603	7·98	0·508	5·89
Valine	0·407	5·86	0·618	9·12	0·436	5·77	0·695	8·07
Cystine	0·167	2·40	0·071	1·04	0·203	2·68	0·145	1·68
Methionine	0·111	1·59	0·104	1·53	0·094	1·24	0·165	1·91
Proline	0·210	3·02	0·232	3·41	0·172	2·27	0·269	3·12
Phenylalanine	0·531	7·64	0·442	6·50	0·585	7·74	0·664	7·71
Tyrosine	0·343	4·94	0·233	3·42	0·339	4·48	0·341	3·96
Histidine	0·154	2·21	0·301	4·43	0·164	2·17	0·184	2·13
Arginine	0·167	2·40	0·167	2·45	0·168	2·22	0·157	1·82
Tryptophan	0·082	1·18	0·100	1·47	0·076	1·00	0·102	1·18
Lysine	0·289	4·16	0·320	4·71	0·317	4·19	0·225	2·61
Ammonia	0·430	6·19	0·421	6·19	0·597	7·90	0·592	6·87

The yields and elemental analyses for these particular samples are reported in Table 2. Ammonia is included in the per cent total. The cystine content is the sum of cystine plus cysteine.

TABLE 4.—AMINO ACID COMPOSITION OF BOVINE CNS WHITE MATTER PROTEOLIPID PROTEIN

Amino acid	FOLCH and LEES (1951)	Present investigation
Leucine, *iso*leucine and phenylalanine	2·49	2·40
Methionine	0·36	0·13
Tyrosine and valine	1·01	1·08
Proline	0·28	0·24
Alanine and glutamic acid	1·81	1·81
Threonine	0·84	0·84
Aspartic acid	0·43	0·40
Serine	0·71	0·47
Glycine	1·14	1·06
Arginine	1·01	0·94
Lysine	0·91	0·89
Histidine	0·64	0·69
Cystine	0·45	0·57
Ammonia	0·89	0·84
Tryptophan	—*	0·21
	12·97	12·57

Nitrogen in constituents as per cent of protein weight
* Not determined

reported by UZMAN (1958) for whole monkey brain (0·88 and 0·98% wet weight) as might be expected if this were a myelin protein. Phosphorus analyses of 0·36 and 0·44 per cent were the only elemental analyses reported by UZMAN for neurosclerin. As we used white matter instead of whole brain, and a different species, there is some question as to the identity of our preparation with UZMAN's neurosclerin. In general, the bovine CNS white matter neurosclerin resembled the proteolipid protein from the same source in its amino acid composition (Table 5 and Table 6).

TABLE 5.—AMINO ACID COMPOSITION OF BOVINE CNS WHITE MATTER 'NEUROSCLERIN'

Amino acid	μmole/mg	% total amino acids
Aspartic acid	0·260	3·83
Glutamic acid	0·370	5·45
Glycine	0·677	9·97
Alanine	0·770	11·34
Leucine	0·708	10·43
*iso*Leucine	0·322	4·74
Serine	0·301	4·43
Threonine	0·527	7·76
Valine	0·387	5·70
Cystine	0·146	2·15
Methionine	0·109	1·60
Proline	0·187	2·75
Phenylalanine	0·523	7·70
Tyrosine	0·344	5·06
Histidine	0·155	2·28
Arginine	0·172	2·53
Tryptophan*	0·076	1·11
Lysine	0·298	4·39
Ammonia†	0·455	6·70

* Determined separately after alkaline hydrolysis
† Included in the total

TABLE 6.—TOTAL RESIDUES OF GLYCINE, ALANINE, LEUCINE AND ISOLEUCINE IN PROTEOLIPID PROTEIN AND NEUROSCLERIN

Material	Percentage of total
Whole monkey brain (UZMAN & ROSEN, 1958)	45·14* and 49·90*
Whole mouse brain (UZMAN & ROSEN, 1958)	48·31*
Bovine CNS white matter neurosclerin	39·11†
Bovine CNS white matter proteolipid	39·73†
Bovine spinal root proteolipid	37·94†
Human CNS white matter proteolipid	39·32†
Human spinal root proteolipid	35·16†

* %of total amino acids excluding tryptophan (which UZMAN and ROSEN did not determine) and proline (which was present only as traces in their preparations). Ammonia is not included.

† % of total amino acids in our analyses (including tryptophan and proline). Ammonia is not included. The inclusion of tryptophan and proline in these calculations makes them about 2·0 per cent lower than those of UZMAN and ROSEN.

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DISCUSSION

It is of interest that this histochemical method demonstrates so well the transition zone between central and peripheral myelin at the cord-root junction. TARLOV (1937) studied this zone with myelin, axonal and glial stains and concluded that the peripheral portion of a nerve fibre has a thicker axon and myelin sheath than the central portion. This thicker peripheral myelin sheath is clearly indicated in longitudinal sections of roots at the transition zone by the heavier staining with osmium and haematoxylin. HOSOKAWA (1959) observed that some peripheral fibres penetrate central to the cord-root junction and he termed this the 'intra-central portion' of a peripheral nerve fibre. LEVI (1906, quoted by HOSOKAWA) stated that the transition zone is intraspinal in the cervical nerves, extra spinal in the lumbar and sacral nerves, and at the cord-root junction in the thoracic nerves. We have not studied the matter extensively but an 'intra-central portion' of peripheral fibres seemed to be a common occurrence in thoracic and lumbar segments of the species we examined (Figs. 2 and 3).

The results reported here are in substantial agreement with those of FOLCH and LEES (1959) on the amount of proteolipid protein in bovine sciatic nerve. They estimated, by acid hydrolysis of the chloroform–methanol extract, that the maximal amount of proteolipid protein would be 0·09 per cent of the wet tissue weight. Our yields of 0·103 per cent for bovine intradural spinal roots are slightly higher and the difference is probably due to the lesser amount of connective tissue in spinal roots. AMADUCCI (1960) estimated the proteolipid content of human spinal roots by acid hydrolysis of the washed total lipid extracts and reported values of 0·44 per cent for anterior roots and 0·47 for posterior roots. FINEAN, HAWTHORNE and PATTERSON (1957) found a very large range in the yield of bovine sciatic proteolipid protein from 0·056 to 0·54 per cent of the wet tissue weight. We are unable to judge the meaning of this, as our yields were within ± 5 per cent of the value stated. BOGANOVE and CLARK (1960) isolated proteolipid A from horse sciatic nerve in a yield of 0·183 per cent but they did not report the nitrogen content of this material. Proteolipid A of bovine CNS white matter contains 3·8 to 4·3% nitrogen (FOLCH and LEES, 1951) and this must be taken into consideration in comparing their results with ours.

The tryptophan content of the bovine spinal root neurokeratin is about 20 per cent more than that of the corresponding proteolipid protein (2·45 and 2·09 per cent of the protein weight respectively). Neurokeratin is also about six times greater in quantity than proteolipid protein in bovine spinal roots. In the CNS the proportion of neurokeratin to proteolipid protein is reversed with the latter being ten times greater. KOENIG (1959) has postulated that neurokeratin and proteolipid protein are the same. In one sense this is true, namely, proteolipid protein and neurokeratin are resistant to trypsin digestion. However, there is no other evidence to indicate that the two proteins are identical in their chemical composition. If they are identical, then the relative proportions of proteolipid and neurokeratin in spinal roots and CNS white matter may be explained on the basis of the efficiency of chloroform–methanol as a solvent for proteolipids. In the CNS chloroform–methanol would be very efficient and only a small amount of unextracted proteolipid protein (neurokeratin) would remain. In the periphery, chloroform–methanol would be less efficient, possibly due to the larger amount of connective tissue and the close investment of the myelin sheaths with the Schwann cells, and the extractable proteolipid protein would be less and the neurokeratin consequently greater in amount.

The present study indicates that CNS white matter contains more than three times the amount of proteolipid and neurokeratin that spinal roots do. This is difficult to explain even when making allowance for the connective tissue in spinal roots. The difficulty is compounded by the fact that peripheral nerve is more heavily myelinated than most central tracts. We have no answer to this enigma but it may be that the bulk of peripheral nerve myelin protein, unlike its CNS counterpart, is not trypsin resistant and therefore not neurokeratin. There is no evidence to support this as yet but the idea merits consideration.

SUMMARY

The tryptophan containing proteins of central and peripheral nerve myelin have been studied histochemically. In CNS myelin this protein is solely proteolipid protein; in peripheral nerve the situation is more complex.

The elemental and amino acid compositions of human and bovine central and peripheral nerve proteolipid proteins and bovine neurosclerin are presented.

A method is indicated for the isolation of peripheral nerve neurokeratin virtually free of collagen and mucopolysaccharides.

Acknowledgements—We are indebted to Mrs. R. CAMILLE CAMPBELL and to Miss MARGARITA STELE for their valuable technical assistance and to Mr. PHILIP BLEICHER for the photography.

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MODE OF REGENERATION OF ACETYLCHOLINESTERASE IN CHOLINERGIC NEURONS FOLLOWING IRREVERSIBLE INACTIVATION*

E. KOENIG† and G. B. KOELLE
Department of Pharmacology, Schools of Medicine,
University of Pennsylvania, Philadelphia 4, Pennsylvania

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THE concept of somato-axonal convection of cytoplasm as a means by which the cell body supports the metabolic needs of the axon is by no means novel, and in fact, was advanced by GOLDSCHEIDER during the era of acrimony surrounding the neuron doctrine in the 1890's (BARKER, 1899). Indeed, the concept may be considered a direct consequence of the neuron doctrine, since one of its basic tenets states that the axon and all its parts, no matter how remote from the cell body, are an integral part of the nerve cell. It is only recently, however, that a number of attempts have appeared to demonstrate that axonal constituents are derived from the perikaryon. Those that have been reported fall essentially into two categories: (i) efforts to establish the general phenomenon of cellulifugal axoplasmic streaming; and (ii) studies designed to demonstrate somato-axonal migration of proteins or specific enzymes.

It was the comprehensive histological studies of WEISS and HISCOE (1948) that established the likelihood that a continuous peripherally directed movement of axoplasm exists. Additional support for this hypothesis based on morphological considerations was obtained recently by VAN BREEMEN, ANDERSON and REGER (1958) and LUBINSKA (1954).

Although WEISS and HISCOE (1948) postulated that axonal proteins are synthesized in the cell body and are carried peripherad by axoplasmic streaming, they offered no evidence to support their contention. More direct evidence for somato-axonal migration of protein was obtained by the use of isotopically labelled amino acids (SAMUELS *et al.*, 1951; WAELSCH, 1958; KOENIG, 1958; VERNE and DROZ, 1960; and MIANI, 1960). In all instances, proximo-distal displacement of labelled protein with respect to time was observed in peripheral nerves.

Studies serving as a basis for somato-axonal migration of preformed enzymes have centred principally around acetylcholinesterase (AChE)‡ (SAWYER, 1946; LEWIS and HUGHES, 1957) and choline acetylase (ChAc) (HEBB and WAITES, 1956). In both the studies by SAWYER (1946) and HEBB and WAITES (1956), the distal accumulation of AChE and ChAc, respectively, was inferred from an observed increase of enzymic activity in the proximal, regenerating stump of previously axotomized peripheral

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† Predoctoral fellow of the Institute of Neurological Sciences. The work reported in this communication served as a basis for a dissertation submitted in partial fulfilment for the degree of Ph.D. by E. KOENIG in the Department of Physiology.

‡ Abbreviations used: acetylcholine, ACh; acetylcholinesterase, AChE; acetyl-β-methylcholine, MCh; butyrylcholinesterase, BuChE; cholinesterase, ChE; choline acetylase, ChAc; di*iso*propylfluorophosphate, DFP; di*iso*propylphosphostigmine, DPS; endoplasmic reticulum, ER; ribonucleic acid, RNA; ribonucleoprotein, RNP; turnover number, TN; 2-diethoxphosphinylthioethyldimethylamine acid oxalate, 217AO.

nerves over that of the normal, contralateral control nerves. The work of LEWIS and HUGHES (1957), on the other hand, was carried out on outgrowing embryonic nerve of the developing toad, *Xenopus laevis*. In all foregoing instances, the nerves under study were in a process of growth.

It is questionable whether a neuron undergoing growth (*i.e.*, nerve regeneration, embryogenesis) is equivalent in its metabolic dynamics, disposition of enzymes and biosynthetic systems to a normal fully differentiated neuron. This communication presents the results of a study designed to test the hypothesis of proximo-distal convection of preformed enzymes with respect to acetylcholinesterase in nerves where growth is not a factor. A preliminary account of this study has appeared (KOENIG and KOELLE, 1960).

The dual considerations of the proposed protein-synthesizing function of ribonucleoprotein and its apparent anatomical restriction to the cytoplasm, coupled with the observations establishing the phenomenon of somato-axonal convection, would appear to indicate that a given axonal protein is derived from the corresponding perikaryon. Accordingly, if axonal AChE is synthesized solely in the cell body, the following propositions should hold:

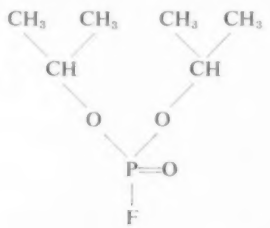
(A) After effective, irreversible inactivation of AChE of the total neuron:

(1) There should be a gradient of AChE activity extending in a proximo-distal direction along the entire somato-axonal course of the neuron during the early phases of AChE regeneration, and (2) the additional procedure of chronic, local suppression of AChE regeneration in a nucleus located in the central nervous system should reduce markedly the total enzyme regenerated in its peripheral nerve for the corresponding interval of time of suppression.

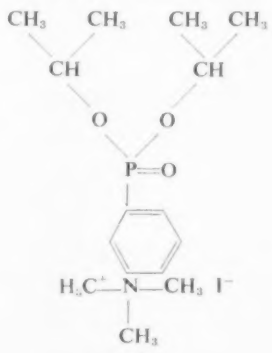
(B) After effective, irreversible inactivation of peripheral AChE only:

(1) The rate of AChE regeneration should be significantly greater in peripheral nerve trunks having their somata located in the central nervous system than in the previous situations (A-1 and A-2).

Propositions A and B can be tested experimentally by administering under appropriate conditions two corresponding irreversible organophosphorus inhibitors: di*iso*propylfluorophosphate (DFP) and di*iso*propylphosphostigmine iodide (DPS).



DFP



DPS

The former is highly lipoid-soluble and therefore penetrates the brain very readily, whereas DPS has a quaternary ammonium group which impedes its penetration across the haematoencephalic barrier. As indicated below, both compounds yield the

identical dialkylphosphoryl residue which phosphorylates the active centres; the enzymically inactive product shows no evidence of spontaneous hydrolytic reactivation (HOBBIGER, 1951; DAVISON, 1955; WILSON and MEISLICH, 1953), hence the reappearance of the enzyme *in vivo* is assumed to represent newly synthesized AChE (BURGEN and HOBBIGER, 1951).

METHODS

General experimental procedure. Adult cats weighing over 2·5 kg were anaesthetized with sodium pentobarbitone (35 mg/kg, i.p.) and subsequently given atropine (5 mg/kg, i.p.). An hour after anaesthetization, either DFP, 40 μmoles (7·36 mg/kg) (series A-1 and A-2), or DPS, 60 μmoles (19·7 mg/kg) (series B-1) was injected *via* the femoral vein. Since DFP is very unstable in an aqueous medium (the half-life of an aqueous solution is approximately 10 hr), the stock solution of DFP was made up in propylene glycol and diluted to the appropriate concentration with 0·9% NaCl solution immediately before injection. Stock solutions of DFP were prepared freshly every two weeks. Since DPS is quite stable, it was made up directly in 0·9% NaCl solution to the appropriate concentration and stored in the refrigerator. In addition to the one systemic injection of DFP, the cats for testing proposition A-2 received intraventricular (4th ventricle) injections of DFP daily through an indwelling cranial cannula. The local intraventricular injections of DFP permitted chronic suppression of the neuronal somal accumulation of AChE in the hypoglossal nucleus without inhibiting appreciably the AChE in the peripheral hypoglossal trunk. The experimental animals were killed at intervals of 1, 2, 3, 5, 10, and 15 days in the A-1 series; 3, 5, and 10 days in the A-2 series; and 1, 3, 5, and 10 days in the B-1 series, after the systemic administration of the anti-ChE agent. All animals were killed by the perfusion of 0·9% NaCl solution through the heart after anaesthetization with sodium pentobarbitone. The structures under study were excised, placed in 0·9% NaCl solution, and stored in the deep freeze until assayed for AChE.

Method for intracranial injections. The components of the cannula system were machined from syringe needles and are depicted in Fig. 1*a*. The device consisted simply of a fixed length cannula sleeve or guide (syringe cannula sleeve), stylet (sleeve stylet), and syringe cannula. The syringe cannula sleeve was secured firmly to the calvarium with quick-setting acrylic dental cement and had a constant depth of penetration of about 21 mm. The sleeve stylet screwed into the syringe cannula sleeve and was removed only immediately before the syringe cannula was inserted for injection. Either stylet or syringe cannula, when positioned in the cannula sleeve, emerged slightly beyond the sleeve tip.

Intraventricular injections of DFP were instituted 1 day after the systemic administration of the usual dose (40 μmole/kg) of DFP. A total amount of 6 μmoles (1·10 mg) of DFP in a volume of 0·1 ml of peanut oil was introduced into the 4th ventricle through the syringe cannula with a tuberculin syringe. All cats were routinely given a protective dose of atropine sulphate (5 mg/kg, i.p.) at least 10 min before the intracranial injection of DFP. Finally, all cats were killed 24 hr after their last intracranial injection.

Structures under study. Selection of structures was based on obtaining a broad representative profile of AChE-regeneration in neuronal somata, axons, axonal terminals, and a non-neuronal tissue. The structures isolated for study were as follows: (i) the hypoglossal nucleus (*i.e.*, cholinergic somata); (ii) a length of 8–10 cm of peripheral hypoglossal nerve beginning with the emerging rootlets (*i.e.*, cholinergic axons), divided into two successive segments of 2 cm each, and a third segment constituting the remainder; (iii) a length of 6 cm of distal cervical sympathetic nerve (*i.e.*, cholinergic axons), divided into two successive segments of 3 cm each; (iv) the superior cervical ganglion (*i.e.*, cholinergic axonal terminals); (v) the ciliary ganglion (*i.e.*, peripheral collection of cholinergic somata); (vi) the inferior oblique muscle of the eye (*i.e.*, a non-neuronal tissue). Wherever applicable, the connective tissue mantle (*e.g.*, epineurium) was removed before the structure was homogenized.

Shaking apparatus. Total volumes of 0·06 ml were incubated in capped, thick-walled test tubes (75 × 10 mm) secured to a specially designed mixing vibrator, schematically represented in Fig. 1*b*. The reaction vessels were clipped at right angles by fuse clips to the rim of a vibrating lucite plate. The vibratory motion was produced by a constant-speed motor with two eccentrically oriented, adjustable brass discs on its drive shaft. The lucite plate was suspended from the motor by a brass rod and the entire assembly was mounted to a base board by a flexaframe support. The magnitude of vibratory motion could be controlled by adjusting the axis of eccentricity of one brass disc relative to that of the other and/or the length of the supporting brass rod.

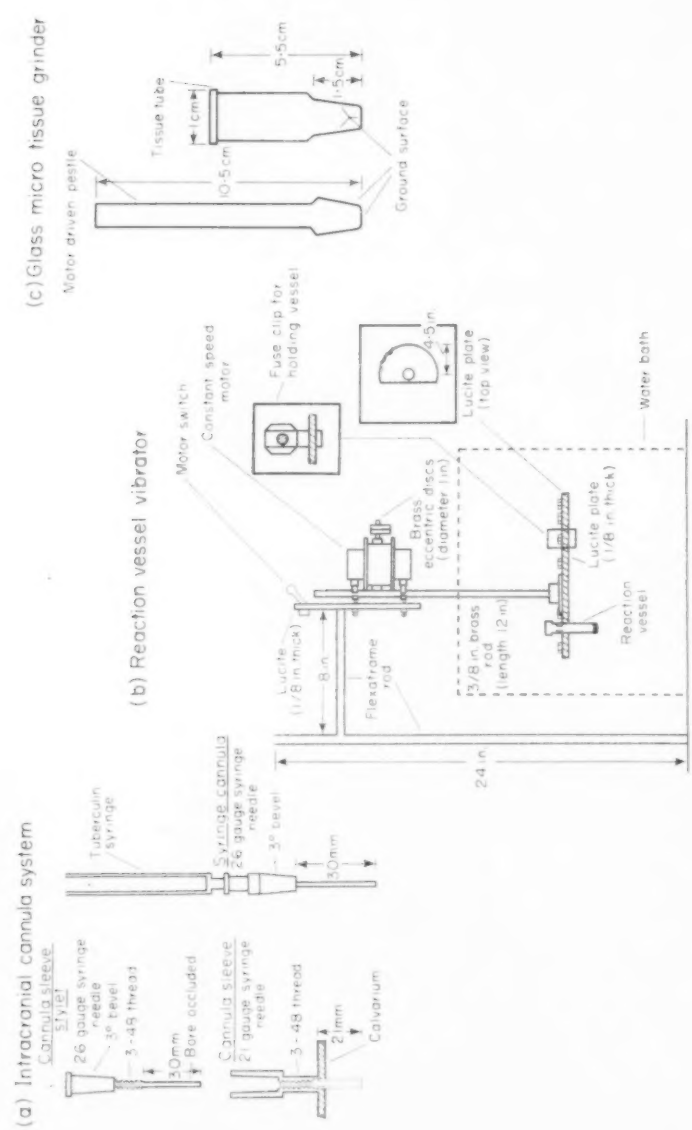


FIG. 1.—Especially designed (a) intracranial cannula system, (b) reaction vessel vibrator, and (c) glass micro-tissue grinder used at various stages of the experimental and assay procedures.

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Assay for AChE activity. For purposes of the present study, a modification of the BONTING-FEATHERSTONE (1956) method for cholinesterase determination was employed, as outlined in Table 1. The original paper should be consulted for details regarding composition of reagents and procedure. In addition to altering volumes and concentrations of various reagents, acetyl-β-methylcholine (methacholine, MCh), a specific substrate for AChE (MENDEL and RUDNEY, 1943), was substituted for acetylcholine (ACh). Final substrate concentration was 0·015 M. The amount of substrate hydrolysed during incubation was not permitted to exceed 15 per cent of the initial total by previously adjusting the concentrations of tissue in the homogenates. The samples were weighed rapidly to the nearest 0·05 mg on the hook of a VDF microtorque balance, with the exception of the inferior oblique muscle which was weighed to the nearest 0·1 mg on an analytical balance. The structures were then homogenized in barbitone buffer with a motor driven micro tissue grinder immersed in an ice bath. The micro tissue grinder (see Fig. 1*c*) enabled the homogenization of volumes as small as 30 μl with a

TABLE 1.—HYDROXAMIC ACID METHOD ADAPTION AND ITS COMPOSITIONAL DELINEATION*

Reagent	Stock	A Reaction sample	B Enzyme blank†	C Reagent blank	D Substrate blank
‡ Tissue homogenate		20 μl	—	—	20 μl
‡ Barbitone buffer		—	20 μl	60 μl	40 μl
‡ Methacholine	0·0225 M	40 μl	40 μl	—	—
NaOH	7·0 N				
plus		40 μl	40 μl	40 μl	40 μl
NH_2OH	6·0 M				
HCl	0·25 N				
plus		1·0 ml	1·0 ml	1·0 ml	1·0 ml
$FeCl_3$	0·067 M				

* See BONTING and FEATHERSTONE (1956) for composition of reagents.
† Standard has identical composition, but is frozen during incubation period.
‡ Incubation mixture.

maximal loss, because of surface adsorption, of 6 μl. Small volumes were transferred with Linderstrom-Lang volumetric pipettes, and reaction mixtures were incubated at 37·5 $\pm$ 0·5° for a period of 2 hr in the case of peripheral hypoglossal nerve, and 1 hr for all other structures. The two types of incubations were carried out separately and on different days. After conversion of the residual unhydrolysed ester to the stable acethydroxamic acid with alkaline hydroxylamine at the end of the incubation period, ferric chloride was added to sets of 4 vessels, centrifuged at 3,000 **g** for 10 min or more, decanted, and the absorbance of the supernatant liquid was read in a Beckman DU spectrophotometer with a photomultiplier at 500 mμ. This operation took approximately 20 min and minimized loss of colour intensity (1 per cent loss in 3 hr according to BONTING and FEATHERSTONE, (1956)). The reagent blank absorbance (Table 1, column C) was subtracted from those for the other reaction mixtures, after which the substrate blank (D) absorbance was subtracted from that of the assay mixture (A). The difference between the corrected absorbances for enzyme blank (B) and assay mixture (A), representing the loss of absorbance because of enzymic hydrolysis, was divided by the value for absorbance of frozen standard per total amount of substrate, yielding the number of μmoles of substrate hydrolysed.

The question of retention within the tissue of uncombined inhibitor which might react with AChE during homogenization and result in false low values was investigated with a related anti-ChE agent, 2-diethoxphosphinylthioethyldimethylamine acid oxalate (217 AO), in an earlier study in this laboratory (KOELLE and STEINER, 1956). Following the intravenous injection of that compound, no uncombined agent could be detected in the brain after 1 hr. By a different method, KEWITZ (1957) found free DFP in the brain 10–15 hr after intravenous injection, but not after 24 hr. Since this was the shortest time in the present study between the injection of DFP and killing of the animal, it is assumed that this factor did not affect the results obtained. This of course does not exclude the possibility of the presence of free DFP at immediate central sites following its intraventricular injection

(part A-2); after the intraventricular injection of 217 AO, uncombined agent was found in the brain for several days (KOELLE and STEINER, 1956). However, the absolute values obtained for the AChE of the hypoglossal nucleus in these experiments have little direct bearing on the conclusions reached from the total study.

RESULTS

Acetylcholinesterase of control tissues

The structures used in the present study, with their mean wet weights and AChE activities are shown in Table 2.

TABLE 2.—AVERAGE WEIGHTS AND CONTROL ACTIVITIES FOR ACETYLCHOLINESTERASE IN STRUCTURES STUDIED

Tissue	Mean wet weight* (mg)	AChE activity† (μmole/mg/hr)	Deviation of mean (%)
XII Nucleus	1·8 ± 0·2	354 ± 11	3
XII Nerve			
Segment 1 (0–2 cm)		27 ± 3‡	11
Segment 2 (2–4 cm)		25 ± 3‡	12
Segment 3 (4-remainder)		28 ± 4‡	14
Total (*ca.* 10 cm)	38·6 ± 3·2		
Cervical Sympathetic Nerve			
Distal segment (3 cm)		212 ± 67	31
Proximal segment (3 cm)		210 ± 45	21
Total (*ca.* 6 cm)	9·4 ± 1·8		
Superior Cervical Ganglion	13·4 ± 2·8	397 ± 119	30
Ciliary Ganglion	2·0 ± 0·5	670 ± 39	6
Inferior Oblique Muscle	77·5 ± 16·2	216 ± 28	13

* Mean of at least 20 separate samples (control and experimental).
† Mean of at least 4 normal control animals.
‡ μmole/mg/2 hr. S.D. $= \sqrt{\frac{\Sigma (\bar{x} - x)^2}{n}}$, when $n > 9$; otherwise, the denominator is $n - 1$.

There are several points worth noting with respect to the AChE activities of the various structures. When the total activities of the hypoglossal nucleus and nerve are calculated separately and compared, it is found that the nucleus has about 15 per cent more total enzyme. However, the length of hypoglossal nerve which was isolated by no means represents the entire length of peripheral nerve; a 1-cm segment which courses through the brain stem, some small, lateral bundles which innervate extrinsic tongue muscles, as well as all distal-most portions of the nerve were not included. It is likely, therefore, that the peripheral nerve contains, in absolute terms, more AChE than does the nucleus.

Another notable comparison is the relative activities of the two peripheral nerve trunks. On a unit wet basis, the cervical sympathetic nerve trunk is 15 times more active than the hypoglossal nerve. Two considerations may explain this difference. First, the hypoglossal trunk contains A fibres (BLOM, 1960), whereas the sympathetic trunk probably contains only B and C fibres (HILLARP, 1960). Thus, the relatively greater amount of myelin in the former would 'dilute' its enzymic activity. FOLEY and

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SCHNITZLEIN (1957) found 7685 ± 1450 fibres in cross-sections of the cervical sympathetic trunk in cats, while BLOM (1960) counted 4866 ± 354* fibres in the proximal segment of the cat hypoglossal trunk. Thus, the cervical sympathetic trunk has almost twice the number of fibres as the hypoglossal nerve. However, despite the importance of the two aforementioned factors, there is reason to believe that they cannot account entirely for the large difference in enzymic activity (*vide infra*). The relatively large standard deviation for AChE activity in the cervical sympathetic trunk, on the other hand, probably can be attributed to (i) the correspondingly high standard deviation for its fibre content, as noted above, and (ii) a rather wide variation in descending postganglionic fibres (*viz.*, 145–1310 fibres (FOLEY, 1945)), which are chiefly adrenergic and contain very little AChE. Likewise, variations in the number of accessory ganglion cells and ganglia, scattered along the course of the cervical trunk (FOLEY, 1945), would contribute significantly to the large standard deviations of the activities of both the sympathetic trunk and its site of termination, the superior cervical ganglion. The standard deviations of AChE activity for all other structures, on the other hand, do not exceed 14 per cent of the mean enzymic activity.

Experiment A-1

The first series of experiments was designed to determine whether a proximo-distal gradient of regenerated AChE activity, developing with time, could be demonstrated after inactivation by DFP. The neuronal system selected for primary study was the hypoglossal nucleus and its peripheral nerve trunk. The choice was made on the basis of (i) the accessibility of a relatively homogeneous population of cholinergic cell bodies and axons, and (ii) the relative absence of centripetal sensory fibres. Intrinsic disadvantages of the hypoglossal system, are (i) the already mentioned low order of activity of the peripheral nerve segments, and (ii) the unavailability of axonal terminals, which would be necessary to complete the nerve unit. Both were minimized, for the most part, by selecting for additional study the enzymically active cervical sympathetic nerve trunk (see Table 1), and the superior cervical ganglion in which approximately 80 per cent of the total AChE resides in the preganglionic axonal terminals (SAWYER and HOLLINGSHEAD, 1945; KOENIG, unpublished). At least three animals were used for each time-interval following DFP injection.

At all time-intervals examined, AChE activities of the three segments of the hypoglossal nerve were essentially equivalent (Fig. 2*a*) and within the standard deviations of each other, with no demonstrable proximo-distal difference. Furthermore, AChE activity returned to control levels by 10 days.

The hypoglossal nucleus, on the other hand, attained only 70 per cent of the control value by 15 days (Fig. 2*b*). On a percentage basis, AChE activity returned at about the same rate in the hypoglossal nucleus as in the peripheral nerve up to the 5th day, but subsequently the rate was markedly less in the hypoglossal nucleus. It can be said, therefore, that there was no apparent proximo-distal gradient of AChE activity in evidence along the peripheral length of the hypoglossal nerve trunk at the time intervals studied. There was a significant difference between the whole trunk and the nucleus after 5 days following AChE inactivation but this difference was in the opposite direction from a somato-axonal gradient.

The segments of the cervical sympathetic trunk and the superior cervical ganglion

* The S.D. was calculated from BLOM's published data by one of the present authors (E. K.).

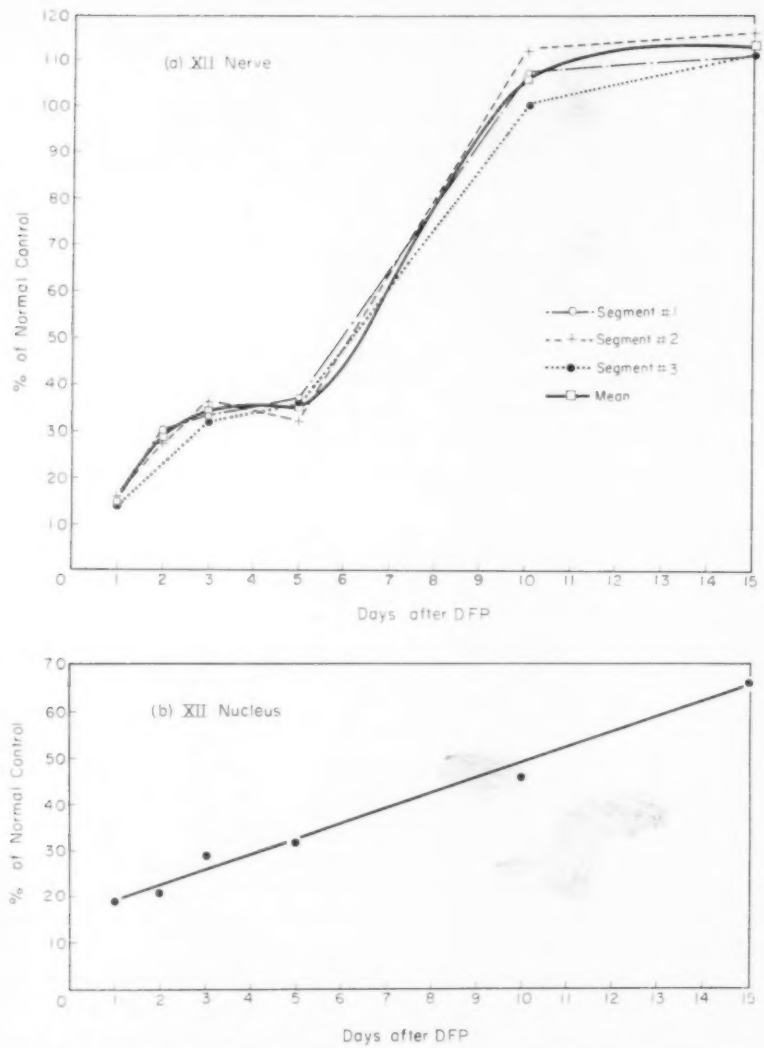

FIG. 2.—Mean relative rates of AChE regeneration in (a) three successive segments of hypoglossal nerve, and (b) hypoglossal nucleus, at 1, 2, 3, 5, 10, and 15 days after DFP administration.

show a pattern of AChE regeneration in the nerve (Fig. 3) not unlike that of the hypoglossal nerve. It is uncertain, however, whether the apparent diphasic patterns in the peripheral nerve structures are real, since the samples were relatively few in number. Nevertheless, the findings with respect to the two segments of the cervical sympathetic and superior cervical ganglion also appear to indicate that no proximo-distal gradient was present in peripheral nerve. Although the curve representing the rate of AChE-regeneration in the superior cervical ganglion appears to 'dip' at 15 days, the small number of samples and the wide variations in control values (see Table 1) argue against assigning any significance to this.

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The ciliary ganglion and inferior oblique muscle were included in the present study for comparative purposes, and their rates and patterns of regeneration are presented in Figs. 4*a* and 4*b*. The patterns of AChE regeneration in both structures over the period studied appear to be quite similar, but the relative percentages of enzyme regenerated in the two structures by 15 days, after DFP injection, were slightly different; the ciliary ganglion, like the hypoglossal nucleus, attained a level that was 70 per cent of the control, whereas in the inferior oblique muscle, the enzyme reached 90 per cent of control level.

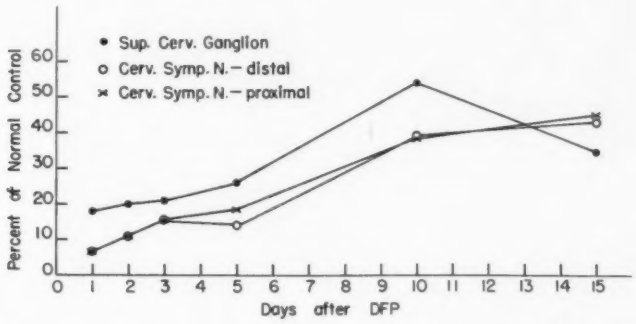

FIG. 3.—Mean relative rates of AChE regeneration in two successive segments of distal cervical sympathetic nerve and superior cervical ganglion at 1, 2, 3, 5, 10, and 15 days after DFP administration.

Acetylcholinesterase is localized both in preganglionic terminals and in all ganglion cells of the ciliary ganglion (KOELLE, 1955; KOELLE and KOELLE, 1959); the latter elements presumably contain the preponderance of the total ganglionic AChE, although no quantitative data are available on denervated ciliary ganglia. Most of the AChE in the inferior oblique muscle, on the other hand, is associated with the subneural apparatus within the endplate region (COUTEAUX, 1958). There is probably a small contribution by the motor nerve terminals to the total AChE activity of the muscle, since the terminal portions of the axons remain unavoidably after dissection.

To make direct comparisons of the absolute rates of AChE regeneration at different sites, it is necessary to calculate the number of functional centres, or esteratic sites, regenerated per mg of tissue. This in turn requires calculation of the turnover number, or TN. The TN represents the number of molecules of ACh hydrolysed per active site of enzyme per min. The number of active sites per mg of tissue has been determined for AChE from various sources by calculating the amount of ^{32}P-labelled DFP in combination with a given amount of completely inhibited purified AChE. With this information, *i.e.*, the number of molecules of labelled DFP/mg AChE (i.e. the number of esteratic sites/mg) in addition to the hydrolytic rates for ACh (molecules of ACh/mg/min), the TN can be derived.

Two TN values for AChE have been reported. MICHEL and KROP (1951) calculated a TN of $4{\cdot}9 \times 10^5$ molecules of ACh/min/esteratic site for purified eel AChE, while COHEN *et al.*, (1955) suggested a value of $2{\cdot}95 \times 10^5$ for bovine erythrocyte AChE. The latter TN appears to be more accurate, since it is corrected for non-specific binding of DFP, and has therefore been used in our calculations. While it cannot be assumed that the same absolute TN value would necessarily apply to the

AChE of cat nervous tissue, the use of this figure here seems justified on the basis of the close approximation of the two values quoted above for AChE's from such widely divergent sources. However, it must be emphasized that the figures obtained from the present calculations represent only approximations of the absolute TN values.

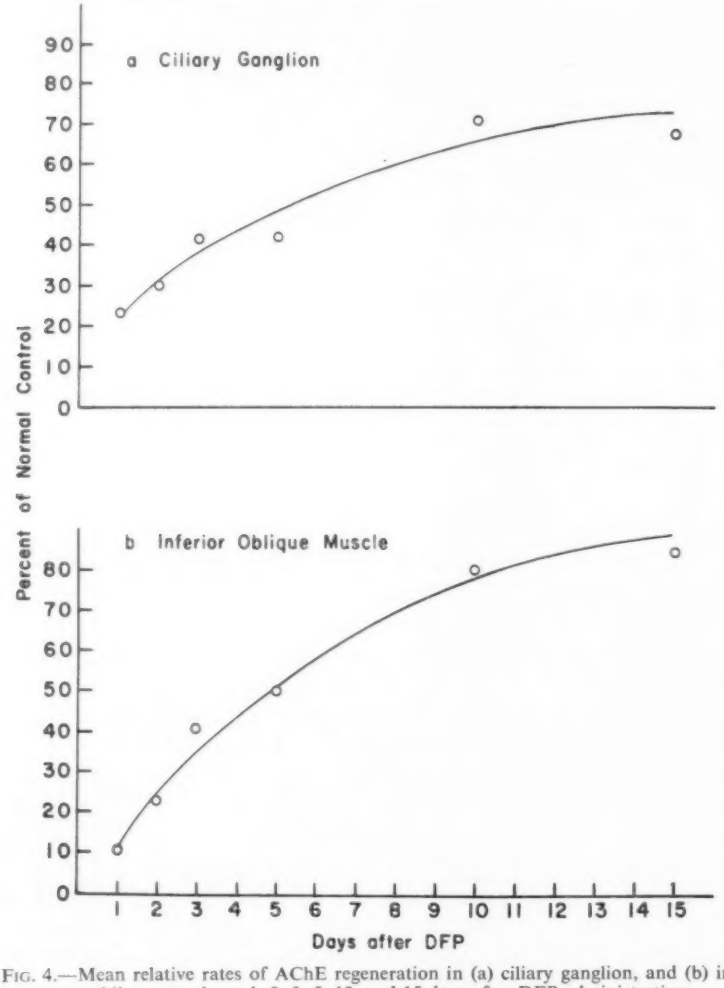

FIG. 4.—Mean relative rates of AChE regeneration in (a) ciliary ganglion, and (b) inferior oblique muscle, at 1, 2, 3, 5, 10, and 15 days after DFP administration.

In the present series, the number of esteratic sites per mg of tissue has been calculated for each structure (Table 3) after conversion of the mean hydrolytic rates of each structure for MCh to ACh-equivalents (ACh is hydrolysed approximately 1·8 times as rapidly as MCh by the AChE of cat brain homogenate (KOELLE, 1957)). The structures are ranked in descending order according to the number of esteratic sites/mg of control tissue. These values bear no relation to the concentrations of esteratic sites in the neurons or their processes, since it is not possible to calculate the proportion of non-neuronal tissue from the data available.

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Table 3 reveals several points of interest. First, for the time interval of 1–15 days, the ciliary ganglion and the hypoglossal nucleus appear to be equally capable insofar as both structures have regenerated 46 per cent of their relative control number of esteratic sites. Regeneration ratios for other time intervals for these same structures are also reasonably close, with the exception of the 10-day interval, when values for the ciliary ganglion are widely scattered. A plot of the relative values for each structure

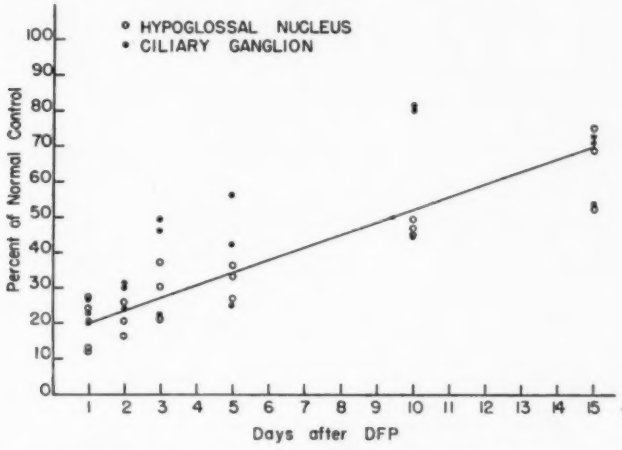

FIG. 5.—Distribution of relative values of AChE regenerated at 1, 2, 3, 5, 10, and 15 days after DFP administration.

TABLE 3.—QUANTITATIVE SURVEY OF ESTERATIC SITES IN STRUCTURES UNDER STUDY FOR CONTROL AND REGENERATED ACETYLCHOLINESTERASE

Tissue	Esteratic sites/mg Control	Esteratic sites/mg Regenerated (1–15 days)	Regeneration ratio* (1–15 days)
Ciliary ganglion	410×10^3	190×10^3	0·46
Superior cervical ganglion	243×10^3	$61{\cdot}4 \times 10^3$	0·25
Hypoglossal nucleus	217×10^3	100×10^3	0·46
Inferior oblique muscle	132×10^3	99×10^3	0·75
Cervical sympathetic nerve	129×10^3	$47{\cdot}3 \times 10^3$	0·37
Hypoglossal nerve	$8{\cdot}5 \times 10^3$	$7{\cdot}8 \times 10^3$	0·92

* Regenerated/Control

(Fig. 5) indicates that they overlap and are not significantly different. This similarity with respect to relative AChE regeneration is not unexpected, since both structures are composed chiefly of cholinergic cell bodies.

Second, perhaps more significant, is what is revealed from a comparison of the two peripheral nerve trunks. Here, the regeneration ratios for the 15-day interval (Table 3) is 0·92 for the hypoglossal nerve, in marked contrast to 0·37 for the cervical sympathetic trunk. However, when the absolute number of esteratic sites per 'unit axonal equivalent' (*i.e.*, unit lengths of nerves equated on the basis of respective fibre contents) for each of the peripheral nerves is examined, the difference becomes vanishingly

small. Thus, a 1-cm length of cervical sympathetic, on the average, weighs approximately 1·5 mg (58 samples), while a comparable length of hypoglossal nerve weighs about 6·0 mg (42 samples). The number of esteratic sites/mg regenerated in both trunks for each interval is converted into the number of esteratic sites/cm by multiplying the former values by the respective conversion weight factors for each nerve. The relative fibre content of the latter, per unit cross-section, is 0·6 that of the former. When both trunks are equated with respect to fibre contents, and the numbers of

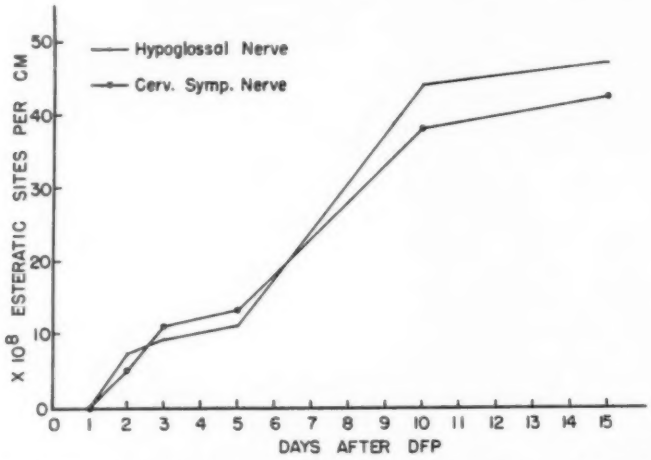

FIG. 6.—A comparison of cumulative averages of esteratic sites regenerated from 1 to 15 days per cm calculated for axons of the hypoglossal and cervical sympathetic nerves (see text for explanation).

esteratic sites regenerated for the various time intervals per unit length are calculated, it becomes evident that the rates of regeneration of esteratic sites, per unit axonal length, in both nerves are approximately equivalent (Fig. 6). It is noteworthy that the agreement between the two nerves is as close as it is, in view of the gross extent of the calculations involved and the operating factor of large biological variation.

Of incidental interest is the calculation which indicates that the fibres of the normal cervical sympathetic nerve have more than twice the number of esteratic sites per cm as those of the normal hypoglossal nerve. This comparison precludes the factor of 'myelin dilution' from consideration (*vide supra*).

It is apparent, from the results thus far presented, that AChE is regenerated in a spatially uniform manner, and in equivalent absolute increments, for the two nerves studied. In the two cholinergic cell-containing structures (*viz.*, hypoglossal nucleus and ciliary ganglion), on the other hand, AChE regeneration appears to proceed at equivalent relative rates as is evident in Fig. 5. Unfortunately, there is no basis for comparing the absolute regeneration of AChE in the subneural apparatus of muscle with that of peripheral nerve. Finally, the character of the regeneration in the superior cervical ganglion (see Table 3, Fig. 3) is not unlike that for peripheral nerve but again no direct means of comparison is possible.

Experiment A-2

The purpose of this series was to determine whether or not chronic suppression of

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somal AChE regeneration would suppress concomitantly the regeneration of AChE in the peripheral nerve trunk. This would appear to be a reasonable corollary of the assumption that the regenerated peripheral AChE is derived from the cell body.

The percentage of AChE regeneration in the A-2 series is expressed (Fig. 7*a*) relative to the mean values for the 'DFP-treated controls' taken from the A-1 series (*i.e.*, cats which received the same intravenous dose of DFP, but no intraventricular injections); the latter are regarded here as the 100 per cent values for the corresponding time intervals. In each of the 3 experimental cats (Fig. 7*a*) the AChE activity

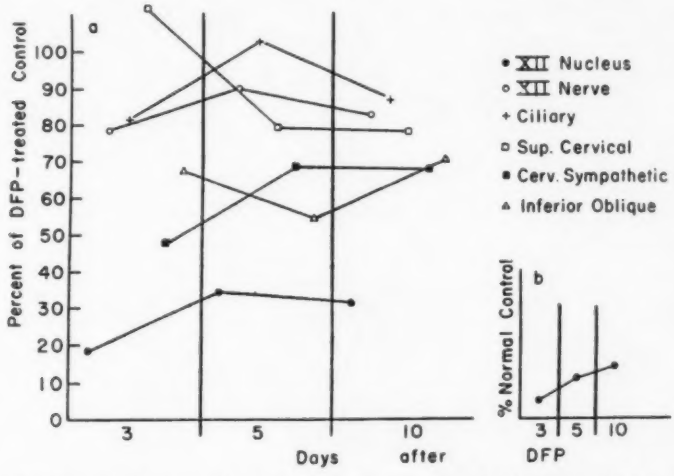

FIG. 7.—Percentage regeneration of AChE after one systemic injection and daily intraventricular injections of DFP, at 3, 5, and 10 days (a) in various structures, relative to DFP-treated controls (animals receiving the same single intravenous injection only), and (b) in the hypoglossal nucleus, relative to normal untreated controls (no DFP). The animals were killed 24 hr after the last intraventricular injection.

of the hypoglossal nucleus never exceeded 34 per cent of that of the mean for the same structure for the A-1 animals. The AChE activity for the hypoglossal nucleus in the A-2 group never exceeded 15 per cent of the untreated control value (*i.e.*, for cats which received no DFP) at any time during the time intervals studied (Fig. 7*b*). These values for the nucleus might represent the percentage of AChE, relative to normal controls, regenerated in a 24 hr period, since these animals were always killed 24 hr subsequent to their last intracranial injection. However, as indicated under Methods, the possibility that free DFP was retained at this site cannot be excluded.

Despite the significant suppression of the AChE of the nucleus, the peripheral hypoglossal nerve regenerated 80 per cent or more of the AChE regenerated in the DFP-treated animals of the A-1 series for comparable time intervals. Enzyme levels in other structures, particularly the cervical sympathetic trunk and the inferior oblique muscle, were suppressed to variable extents by intraventricularly injected DFP. This suggests that the passage of some of the inhibitor into the systemic circulation was responsible for the relatively reduced values in all the peripheral structures, including the hypoglossal trunk.

The results of Experiment A-2 are in agreement with the findings of Experiment A-1, and therefore tend to substantiate them. Considered together, the results indicate

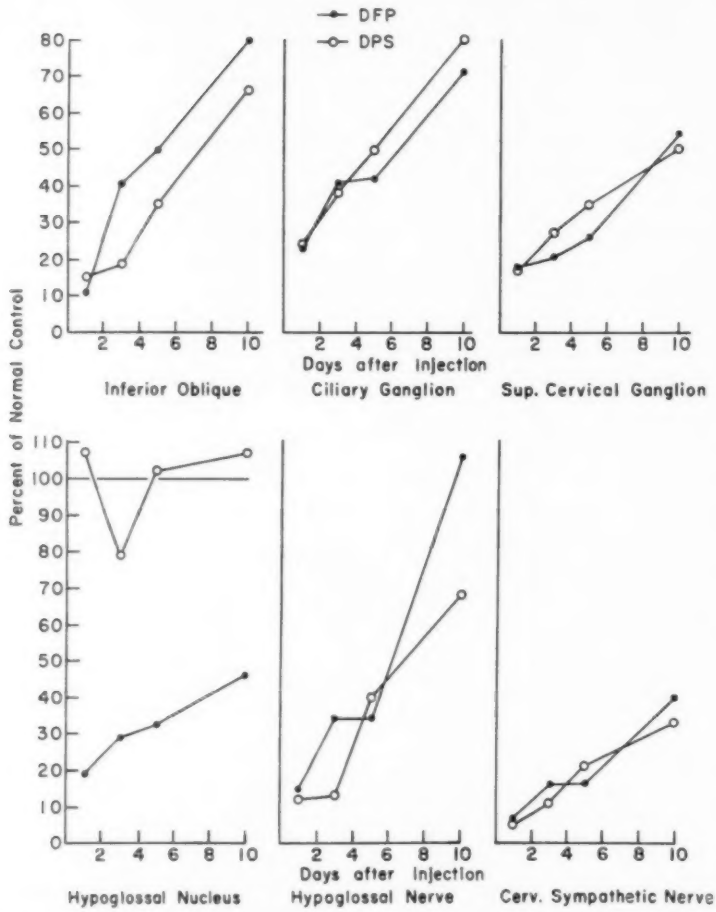

FIG. 8.—Comparison of relative rates of AChE regeneration after DFP and after DPS at 1, 3, 5, and 10 days following adminstration.

strongly that the regeneration of AChE in the peripheral nerve fibres is a process which is essentially independent of the regeneration of AChE in the soma.

Experiment B-1

The experimental test of proposition B-1 requires that the AChE in the peripheral hypoglossal nerve be inhibited selectively without affecting significantly the enzyme in the nucleus. This was found to be accomplished satisfactorily by the intravenous injection of the quaternary anti-ChE agent, di*iso*propylphosphostigmine iodide (DPS). Under these experimental conditions, an accelerated rate of regeneration of AChE in the peripheral nerve should occur if its regeneration in the periphery is contingent upon the availability of somal enzyme for cellulifugal convection.

The AChE-regeneration curves after DPS injection (Fig. 8) show a fair degree of correspondence in all structures with those obtained after DFP injection from Experiment A-1 save for the expected results with respect to the hypoglossal nucleus.

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Each point on the DPS curves represents the average value for at least 2 cats. Of primary significance is the fact that AChE in the peripheral hypoglossal nerve exhibits no indication of relative acceleration in its rate of regeneration.

Of incidental interest is the efficacy of the blood-brain barrier in preventing the penetration of DPS into the central nervous system. Although the AChE activity of the hypoglossal nucleus ranged between 95 and 120 per cent of control activity at the 1-, 5- and 10-day intervals, the mean activity of 3 cats for the 3-day interval was 79 per cent (74–83 per cent). The significance of this fall on the third day after DPS administration is obscure; however, the number of experimental animals was too small to establish the phenomenon with certainty.

These data are consistent with the overall results obtained in Experiments A-1 and and A-2. It may be stated, in summary, that the available evidence is not consistent with the inference that peripheral nerve AChE is derived from the cell body after irreversible inactivation with DFP or DPS.

DISCUSSION

Of fundamental importance in the present experimental study is the certainty with which the factor of spontaneous dephosphorylation of AChE can be ruled out. This much is clear from *in vitro* studies involving DFP-inactivated AChE: no spontaneous reactivation of the inhibited enzyme has been observed to take place, even in freshly inhibited enzyme preparations (HOBBIGER, 1951; DAVISON, 1955; WILSON and MEISLICH, 1953; BLABER and CREASEY, 1959). The permanent chemical alteration (BERENDS *et al.*, 1959), known as ageing, which evidence indicates is complete in 1 day (CREASEY and BLABER, 1960), renders DFP-inhibited AChE completely resistant to dephosphorylation by any of the available nucleophilic reactivators (HOBBIGER, 1951; WILSON, 1955; HOBBIGER, 1955; DAVIES and GREEN, 1956; DAVISON, 1955; CREASEY and BLABER, 1960). The first order reaction of a loss of an *iso*propyl group from di*iso*propylphosphoryl-AChE was shown convincingly by BERENDS *et al.* (1959) to underlie the phenomenon of ageing. An indication of the strength of the mono*iso*-propylphosphoryl-AChE bond is reflected by the fact that it can be cleaved only by such drastic measures as alkaline hydrolysis (BERENDS *et al.*, 1959). Thus, it would appear that the possible likelihood of a phosphatase dephosphorylating mono*iso*-propylphosphoryl-AChE is remote, albeit not ruled out completely (*vide infra*). Nonetheless, the possibility of *spontaneous* dephosphorylation occurring to any appreciable extent beyond 1 day after DFP administration can be excluded with a considerable degree of certainty.

There are 3 inferences that may be drawn from the results of the present study: (i) proximo-distal convection of all axonal enzyme is not a valid generalization; (ii) the peripheral nerve is capable of restoring functional AChE with apparent independence of the soma after irreversible inhibition by organophosphorus compounds; and (iii) cytoplasmic AChE is not a significant reserve source of axonal AChE under the conditions of the present study. The experimental results which constitute the basis for the aforementioned statements are as follows:

(1) There was no demonstrable proximo-distal gradient of regenerating AChE following irreversible inhibition by DFP or DPS at the time intervals studied.

(2) Return of AChE after DFP-inhibition in each of the two peripheral nerves studied, from two divisions of the nervous system, proceeded *pari passu* at equivalent

absolute rates and amounts, in contradistinction to somal AChE from the same divisions which regenerated at equivalent relative rates.

(3) Peripheral nerve AChE was regenerated, after DFP inhibition, despite a chronically maintained marked suppression of somal AChE by periodic intraventricular administration of DFP.

(4) The rate of peripheral AChE regeneration, after its selective inhibition by DPS, was not accelerated over that following DFP-inactivation, despite the existence of a full somal complement of AChE in the former situation.

These findings are in contradiction to the inference that AChE, as well as other enzymes, migrates distad in the axon after its cytoplasmic synthesis. The hypothesis of cellulifugal convection of AChE was based originally on the assumption that the three-fold increase of AChE in the regenerating, outgrowing peripheral stump of the sectioned sciatic nerve over that of the contralateral control, found by SAWYER (1946), is a phenomenon which takes place normally in fully differentiated, mature neurons. It was supported by the finding that neuronal AChE is separated by a membranous barrier into external and internal fractions (KOELLE, 1957), of which only the former appear to be functional in synaptic transmission (MCISAAC and KOELLE, 1959). The loss of cytoplasmic AChE from axotomized somata, however, demonstrated by SCHWARZACHER (1958) to persist for the apparent outgrowth period, casts serious doubt on the validity of the assumption that all AChE is synthesized in the perikaryon and migrates peripherad under normal circumstances. While it is possible, or even likely, that the AChE appearing in the proximal outgrowing stump of regenerating nerve is derived from the cytoplasm initially, the very drastic procedure of dividing the peripheral axonal process is well known to evoke profound alterations in the internal milieu and economy of the cell. Unfortunately, little in the way of definitive work has been undertaken to elucidate the ultrastructural changes that accompany the 'neuron reaction' to axotomy. Such studies may very well reveal a partial or complete dissolution of the endoplasmic reticulum (ER), release of ribonucleoprotein (RNP) granules (*i.e.*, ribosomes), and a migration of RNP granules and ER into the outgrowing axon where the highest protein biosynthetic activity is probably taking place.

Some support for these suggestions comes from ultraviolet (u.v.) absorption studies of RNA in the nervous system during embryogenesis and regeneration. HUGHES (1955) and HUGHES and FLEXNER (1956) observed that u.v. absorption at 2600 Å was demonstrable throughout the developing neuroblasts, including their axonal processes. However, one of the signs of advancing differentiation of the neuroblasts was the loss of absorbance by the axons preceding the appearance of the classical Nissl pattern. In regenerating cell bodies, EINARSON (1933) reported that during chromatolysis there is an accumulation of basophilic material in and around the axon hillock, which is an area conspicuous for its lack of basophilia and low absorbance at 2600 Å. Thus, it is seen that there is reason to believe that ribosomes do invade the axon during growth (*i.e.*, embryogenesis, regeneration).

An analysis of the possible mechanisms operating in the regeneration of AChE in peripheral nerve after irreversible inhibition by DFP can be only conjectural. Nevertheless, there would appear to be four possible explanations for the regeneration of AChE, which are not mutually exclusive. These may be listed briefly as: (i) spontaneous reactivation resulting from a non-enzymic hydrolysis of the alkylphosphorylated AChE; (ii) enzymic reactivation by a tissue phosphate; (iii) *de novo* synthesis of

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the entire enzyme protein molecule within the axon; and (iv) axonal conversion of an enzymically inactive precursor of active AChE, or of a system for reconstitution of the active centre in alkylphosphorylated AChE.

The question of spontaneous reactivation has been considered already. The available evidence weighs heavily against spontaneous reactivation taking place at all, and particularly after ageing of DFP-inhibited AChE is complete. Hence, it seems safe to conclude that this factor did not operate during the time period of the experimental study (*i.e.*, between 1 and 15 days following administration of DFP).

The possibility of reactivation being promoted by a tissue phosphatase, although unlikely, cannot be excluded quite as easily. Fluorophosphatases which hydrolyse free DFP to unreactive di*iso*propylphosphoric acid have been reported (MAZUR, 1946; MOUNTER, 1955). These dialkylfluorophosphatases are present in various tissues (MOUNTER *et al.*, 1955) and are capable, presumably, of attacking only the fluorophosphoryl bond of freely circulating DFP and related compounds and not the alkylphosphoryl-AChE bond. Phosphoprotein phosphatases have been shown also to be present in various tissues (SUNDARARAJAN and SARMA, 1954; SINGER and FRUTON, 1957) which may include nerves, where phosphoprotein has been demonstrated (SAMUELS *et al.*, 1951). However, since there is neither direct evidence that the phosphatases in general can attack alkylphosphorylated proteins, nor direct evidence that DFP-inhibited AChE can be enzymically reactivated, the occurrence of enzymic reactivation of DFP- or DPS-inhibited AChE appears improbable, although it has not been ruled out.

The subject of *de novo* protein biosynthesis is exceedingly complex. That nucleic acids play required roles in the process is generally accepted at present. The principal objection against attributing *de novo* protein synthesizing ability to peripheral nerve is the lack of demonstrable RNA in axons. Thus no RNA can be detected in the axon by u.v. absorption analysis (NURNBERGER *et al.*, 1952) or by the very elegant ultramicro technique of EDSTRÖM (1956); neither do electromicrographs reveal anything resembling RNP granules in the axon (PALAY and PALADE, 1955). Two contentions, which could conceivably circumvent the foregoing objection, are the unlikely possibilities that mitochondria, which are present in axoplasm, or Schwann sheath cells, or both, may serve as AChE-synthesizing centres. AChE activity is high in the mitochondrial fraction (NATHAN and APRISON, 1955; HOLMSTEDT and TOSCHI, 1959) and it is conceivable that the axoplasmic mitochondria do synthesize AChE, but hardly for 'export'. It is highly questionable whether an AChE molecule, with a minimal molecular weight of 63,000 (MICHEL and KROP, 1951) could penetrate readily the mitochondrial membrane.

Likewise, there is the same difficulty in attributing to the Schwann sheath cells the synthesis of axonal AChE. Again, the AChE molecule would have to penetrate at least one membrane, if not a multimembranous system. Moreover, the Schwann cells have been shown histochemically to contain BuChE but not AChE (KOELLE, 1951).

Finally, the possibility of a stepwise synthetic process for axonal AChE (*i.e.*, AChE precursor) should be examined. The process might entail the synthesis in the perikaryon of an AChE-precursor protein, which is subsequently convected peripherad and converted locally into functional AChE. A case in point is amylase of pigeon pancreas where STRAUB and ULMANN (1957) have shown that a preformed precursor is converted to the active enzyme by the selective incorporation of L-arginine

and DL-threonine. Alternatively, fixed AChE within the matrix of the axonal membrane normally may undergo partial breakdown and reconstitution through the action of local enzymes; this process might result in the replacement of phosphorylated by functional active centres after DFP injection. Proteolytic enzymes, reportedly present in the nervous system (*cf.* RICHTER, 1959) could conceivably participate in such reactions.

Notwithstanding the present negative evidence with respect to AChE, the phenomenon of cellulifugal convection of axoplasmic protein appears well founded. The results of several studies demonstrating the proximo-distal movement of isotopically labelled proteins along the axon (SAMUELS *et al.*, 1951; WAELSCH, 1958; KOENIG, 1958; VERNE and DROZ, 1960; MIANI, 1960) are all consistent with the concept of cellulifugal convection of axoplasm and offer the best and most direct evidence for the phenomenon. Although the results of the present experimental study are inconsistent with the general theory of proximo-distal migration of preformed enzymes, they cannot be considered to refute it. Rather, the results pertaining to AChE regeneration impose certain restrictions on the general applicability of the theory of somato-axonal migration; *i.e.*, they qualify the theory, with AChE as an example of the possible exceptions to it. With respect to AChE itself, it is necessary to emphasize certain aspects of its neuronal localization. First, all available histochemical and biochemical data indicate that the enzyme is firmly associated with membranous structures such as the endoplasmic reticulum and mitochondria in the cytoplasm (NATHAN and APRISON, 1955; TOSCHI, 1959; FUKUDA and KOELLE, 1959), somal membrane (KOELLE, 1957; KOELLE and KOELLE, 1959), axolemmal membrane (BOELL and NACHMANSOHN, 1940; KOELLE, 1957) and axonal terminal membranes (KOELLE, 1957; KOELLE and KOELLE, 1959). The 8 per cent of the total ChE activity of the caudate nucleus found in the supernatant fraction by NATHAN and APRISON (1955) is attributable most likely to BuChE, which is found exclusively in the supernatant fraction of brain homogenates (HOLMSTEDT and TOSCHI, 1959); the former workers reported only ChE activity, and failed to state whether ACh or MCh was used as substrate. Since the foregoing evidence indicates that AChE is firmly associated with certain membranes, it is suggested that axolemmal AChE, as a part of a fixed system, may be more stable than axoplasmic systems. According to this proposal, proteins with enzymic function which form an integral part of the structural matrix of the axolemma may not be synthesized *de novo*, excepting at the time of initial growth processes (*i.e.*, embryogenesis, nerve regeneration). This concept would therefore favour a mechanism of reconstitution to explain the regeneration of peripheral AChE, rather than a mechanism of *de novo* synthesis. The observation that AChE appeared to regenerate uniformly, at dissimilar relative rates but equivalent absolute rates in two different peripheral nerves, coupled with the apparent absence of RNA in the axoplasm argues in favour of linking peripheral AChE regeneration with normal catabolic and restorative processes of the axolemmal structure. The dissimilar absolute, but equivalent relative rates of enzyme return in soma-containing structures studied, on the other hand, might well be a function of *de novo* protein biosynthesis. It is probable that a characterization of the normal mode of catabolism in the axolemma (*i.e.*, partial versus total degradation of membrane protein) would indicate whether conversion of a precursor or reconstitution of the active centre is the principal mechanism for the restorative process.

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Lastly, in addition to some of the implications raised by the present set of experiments with respect to protein metabolism in nerve, is the question of the role of cytoplasmic AChE. If somal AChE is not for axonal 'export', and hence not a reserve for axonal AChE under normal conditions, it is a matter of some interest to ascertain its function, which at present is only conjectural.

SUMMARY

In the present study, an attempt was made to determine whether AChE normally is synthesized in the perikaryon and convected proximo-distally in the axon. Three complementary types of experiments were performed to test the hypothesis. In all three experimental situations, the regeneration of AChE activity after di*iso*propylphosphorylation of the enzyme's active centre(s) was followed in various cholinergic structures of the cat over a period of time.

It was found that AChE regenerated in a spatially uniform fashion and at dissimilar relative rates but equivalent absolute rates in the two peripheral nerves studied. In the two neuronal soma-containing structures, on the other hand, the AChE regenerated at dissimilar absolute, but equivalent relative rates. Furthermore, the results of experiments A-2 and B-1 indicated that the regeneration of AChE in the periphery was proceeding independently of the cell bodies. The two apparent modes of AChE regeneration in axons and cell bodies, respectively, are discussed in respect of the possible mechanisms.

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AMINO ACID AND PROTEIN METABOLISM OF THE BRAIN—VII*

THE PENETRATION OF CHOLINESTERASE INHIBITORS INTO THE NERVOUS SYSTEM OF THE FROG

DORIS H. CLOUET and HEINRICH WAELSCH
New York State Psychiatric Institute and Columbia University,
College of Physicians and Surgeons, New York, N.Y.

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IN THE course of our studies of cerebral protein metabolism (LAJTHA *et al.*, 1957; FURST *et al.*, 1958) the incorporation of [^{14}C]lysine into the proteins of succeeding segments of the sciatic plexus and nerve of the frog was investigated (WAELSCH, 1958; LAJTHA, 1961). It was found that a proximal-distal gradient of specific activity of protein bound lysine developed in time, a gradient which reversed itself after several weeks, the specific activity of the protein bound lysine of the most distal nerve segment being equal to or higher than that of the proximal segments of the nerve and plexus. This finding aroused our interest in the question whether or not the gradient of radioactivity observed is an indication of synthesis of the axonal protein in the perikaryon of the respective nerve cells and transportation distally by axonal flow. In order to extend these studies by measurement of a parameter other than incorporation of an isotopic amino acid, the distribution, and recovery after inhibition, of the total cholinesterases (substrate: acetylcholine) and also of pseudo cholinesterase (butyryl choline) and true or acetyl cholinesterase (acetyl β-methylcholine) were investigated, since changes in enzyme activity may tentatively be taken as a measure of the synthesis or breakdown of the enzyme protein. Such a study by itself or when combined with isotope experiments might provide information as to the origin of the esterases of peripheral nerve, and whether they are synthesized in the cell body and transported in the nerve fibre to the nerve ending or synthesized locally in the fibre itself. Preliminary to such an investigation, the knowledge of the time sequence of inhibition of the enzymes is essential. Therefore, the relative rates of penetration of inhibitors from the cerebrospinal fluid and from blood into peripheral nerve were examined by use of two related compounds with approximately equal *in vitro* cholinesterase inhibitory activity, one an ionized quaternary ammonium compound, the other a lipophilic tertiary amine. The cholinesterase inhibitors were injected either into the subcutaneous lymph sac or into the fourth ventricle of the brain of the frog, and the levels of pseudo and true cholinesterases in brain, spinal cord and nerve were determined at various times to establish the sequence of inhibition, and the route by which the

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A preliminary report of some of the findings has been made in: CLOUET D. H. and WAELSCH H. (1961) *The Regional Chemistry, Physiology and Pharmacology of the Nervous System.* Edited by KETY S. S. and ELKES J., Pergamon Press, New York.

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inhibitor reached the enzyme. In the succeeding paper a study of the recovery of the enzymes after inhibition will be reported.

EXPERIMENTAL

Cholinesterase inhibitors. The pharmacological properties of the quaternary ammonium compound 217MI (phospholine iodide) and its tertiary acid oxalate, 217AO, (both obtained through the courtesy of Dr. R. A. LEHMAN of The Campbell Pharmaceutical Company of New York, N.Y.) have been described by KOELLE and STEINER (1956) who have reported oil/water partition coefficients of 0·00052 for 217MI and 0·112 for 217AO, thereby accounting for the lipophilic nature of 217AO, and the lipophobic nature of 217MI. In *in vitro* inhibition studies in which rabbit brain acetylcholinesterase

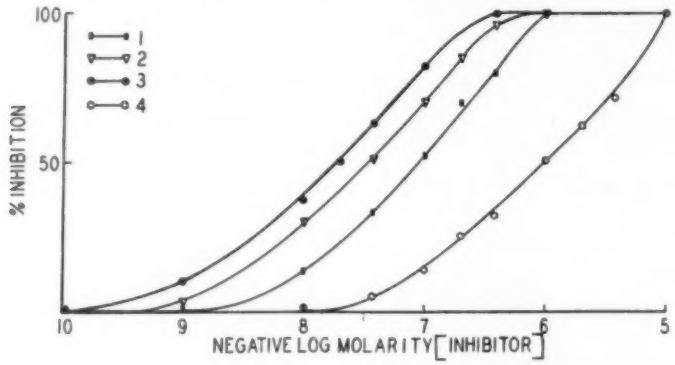

FIG. 1.—The *in vitro* inhibition of cholinesterases

Curve	Inhibitor	Substrate	Tissue	Preincubation (min)
1	217AO	0·5 mM-acetylcholine	Brain	30
2	217MI	0·5 mM-acetylcholine	Brain	30
3	217AO	5 mM-butyrylcholine	Nerve	30
4	217AO	0·5 mM-acetylcholine	Brain	0

In curves 1, 2 and 3, the tissue homogenate and the inhibitor were preincubated together at 37° for 30 min before the addition of substrate. In curve 4 all components were added at the same time.

was preincubated for 30 min with the inhibitor before the addition of substrate, KOELLE and STEINER found the pI_{50} (negative log of molar concentration for 50 per cent inhibition) for 217AO to be 7·11 and for 217MI,7·88. In the assay system described below with frog brain as the source of cholinesterase and a 30 min preincubation of enzyme and inhibitor, the pI_{50} for 217AO was found to be 7·05 and for 217MI 7·45 when acetylcholine was the substrate. When sciatic nerve was the enzyme source and butyrylcholine the substrate, the pI_{50} for 217AO was 7·71 (Fig. 1).

Treatment of frogs and preparation of tissue. The inhibitors were dissolved in frog Ringer solution in a volume such that the total dose was contained in a volume of 0·05 ml. This amount was injected into a frog (*Rana pipiens*) either directly into the fourth ventricle of the brain through the skin and vascular membrane or into the subcutaneous lymph sac. In the control experiments, frog Ringer solution was injected via the same routes. The frog was killed by decapitation, and the brain, spinal cord, sciatic plexus, and sciatic nerves were removed and twice rinsed with the Ringer solution. The spinal cord, plexus, and nerves were each divided into two equal segments, and the two proximal segments of the plexus were combined as were the two distal segments, and the corresponding segments of the sciatic nerves. Each segment or combination of segments was removed, homogenized, and

Abbreviations used: 2-diethoxyphosphinylthioethyltrimethyl ammonium iodide, 217MI; 2-diethoxyphosphinylthioethyldimethylamine acid oxalate, 217AO; acetylcholine AcCh; butyrylcholine, BuCh; acetyl β-methylcholine, MeCh; benzoylcholine, BzCh; ethylbutyrate, EtBu; monobutyrin, MonoBu.

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assayed separately before removal of the next segment. In experiments in which low cholinesterase levels were anticipated, the corresponding samples of two frogs were combined. The tissue was homogenized in a glass homogenizer with close tolerance in a volume of Ringer solution such that the resulting homogenate contained approximately 1 mg of protein/ml. The homogenates were freed of gross particles and fibres by filtration through glass wool and assayed directly.

At the time of decapitation several drops of whole blood were collected and diluted with water to lyse the red blood cells, and portions of this suspension were assayed for cholinesterase activity.

Cholinesterase assay. A modification of the micromethod of Lowry *et al.* (1954) was used. The acid released from the choline ester was measured colorimetrically with phenol red as an indicator. In a 3 ml Beckman cuvette was placed 0·5 ml of indicator solution (0·06% phenol red in 0·03 M-sodium barbitone diluted 1:10 with a diluent solution containing mM-$MgCl_2$ and 0·1 M-NaCl),

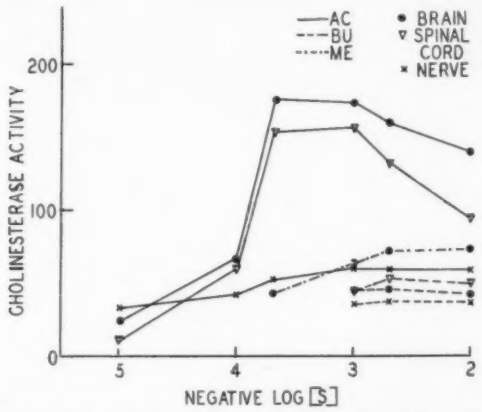

Fig. 2.—The effect of substrate concentration on enzyme activity. Cholinesterase activity is expressed as moles of acid released $\times 10^9$/mg protein/min.

0·2 ml of the diluent solution, 0·1 ml substrate, 0·1 or 0·2 ml of tissue homogenate, inhibitors when added, and water to make a volume of 1·2 ml with a final pH of 8·6. A 9 mm glass plunger reduced the optical path to 1 mm, and nitrogen gas was bubbled through the solution to stir it and to exclude atmospheric carbon dioxide. The optical density was measured at 561 mμ in a Beckman spectrophotometer. For each assay, one of the four cells contained a water blank, one an indicator-tissue blank, one the assay with AcCh, and one with either BuCh or MeCh as substrate. At zero time the tissue was removed from the frog, rinsed, homogenized, and filtered, and the resulting enzyme preparation added to the cells with an elapsed time of about 1·5 min; the readings of optical density were made each minute thereafter for 10 min. The rate of acid released was calculated from standard curves obtained with known amounts of acetic or butyric acid. The initial blank optical density reading was about 0·350, and each decrease of 0·100 corresponded to a release of 4×10^{-7} moles of acid.

Additional buffer-indicator dye systems were adapted to the cholinesterase assay to vary the pH of the medium from 6·4 to 9·4. However, maximal cholinesterase activity was obtained with the phenol red-barbitone system at pH 8·6.

The protein in each portion was measured by the method of Lowry *et al.* (1951). The enzyme activity was expressed as moles of acid released/mg of protein/minute.

The effect of substrate concentration on the assay at pH 8·6 for cholinesterase in the frog nervous system is shown in Fig. 2. The final concentrations of the substrates used in the assay were 5 mM-MeCh and 5 mM-BuCh and 0·5 mM-AcCh.

To establish the relative activities of the various esterases in the frog nervous system, and particularly of the true and pseudo cholinesterases, the activity of nervous tissue homogenates toward various esters was determined (Table 1). The level of true cholinesterase, as measured by the hydrolysis of MeCh was highest in the central nervous system, and of the pseudo enzyme, as measured by the hydrolysis of benzoylcholine (BzCh) in peripheral nerve. Esterase activity was also highest in nerve,

TABLE 1.—THE ESTERASE ACTIVITY OF THE NERVOUS SYSTEM OF THE FROG

Sample	Substrate					
	AcCh	BuCh	BzCh	MeCh	EtBu	MonoBu
Brain	100	12	6	47	6	9
Spinal cord	100	23	17	33	19	12
Plexus	44	30	18	6	18	5
Sciatic nerve	52	36	24	10	30	2
Blood	12	6	4	4	1	5

In this experiment, the substrate concentrations were AcCh = 0·1 mM-acetylcholine, BuCh = mM-butyrylcholine, BzCh = mM-benzoylcholine, MeCh = mM-acetyl β-methylcholine, EtBu = mM-ethyl butyrate, and MonoBu = mM-monobutyrin. The activity of brain cholinesterase was taken as 100 (= 165 μm-moles of acid released per mg protein per min at pH 8·6) and the other values are expressed as percentages of it. The corresponding segments of four frogs were combined for this experiment.

and lipase activity in the central nervous system and blood. Each tissue has at least slight hydrolytic activity toward each of the substrates tested.

Estimation of in vitro *inhibition.* When an inhibitor is injected directly into the brain and a homogenate of the central nervous system is prepared after a short time, the problem arises of assessing the amount of *in vitro* inhibition which occurs during the homogenization and other manipulations by excess inhibitor not bound to the enzyme *in vivo*. When in *in vitro* experiments the substrate and inhibitor were added simultaneously an interval occurred during which the substrate protected the enzyme before replacement by irreversibly bound inhibitor (Fig. 3). When hydrolysis rates for the first 5 min of inhibition without preincubation of inhibitor and enzyme were compared with those after the usual 30 min preincubation, an approximately ten-fold higher concentration of inhibitor was necessary for 50 per cent inhibition (Fig. 1). This was true for human serum pseudocholinesterase and cholinesterase of human erythrocytes as well as for the frog brain enzymes. The resulting increase in the sensitivity of the detection of inhibition and, particularly, the method of distinguishing *in vitro* from *in vivo* inhibition made it advisable to evaluate only the initial rate of hydrolysis in the calculation of enzyme activities, and to use the inflection in the curve at 3–7 min for recognizing the occurrence of *in vitro* inhibition.

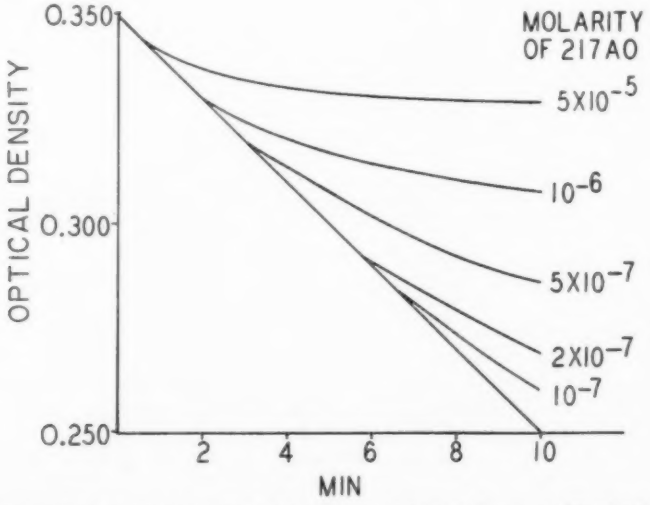

FIG. 3.—The *in vitro* inhibition of cholinesterase by 217AO. Portions of frog brain homogenate containing 0·20 mg protein/portion were assayed with acetylcholine as substrate. The final concentration of 217AO in each assay is shown on the right hand side of the graph.

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The amount of free inhibitor was measured in a separate assay (Table 2). In this assay a control sample, an inhibited sample, and a combination of the two were pre-incubated for 30 min with buffer-dye solution, the diluent, and water, before the pH was adjusted to pH 8·6, and the substrate was added for the assay. If no free inhibitor had been present in the homogenate the sum of the values of the control sample (column 1) and the inhibited sample (column 2) would correspond to the value of

TABLE 2.—THE ESTIMATION OF FREE INHIBITOR LEVELS

Tissue—time	Control	Inhibited	Control plus inhibited	Inhibition (%)
Brain	(1)	(2)	(3)	(4)
45 min	19·2	3·2	20·8	8
60 min	29·6	5·6	36·2	(+2)
Spinal cord (proximal)				
45 min	19·2	3·2	18·0	23
60 min	29·6	9·6	28·0	38
2 hr	34·0	4·8	25·8	38
6 hr	29·6	2·8	23·2	31
1 day	28·8	3·2	26·8	18
2 days	27·2	8·0	34·8	1
4 days	26·8	5·6	32·8	(+2)
10 days	28·8	4·4	32·2	2
Spinal cord (distal)				
45 min	19·2	3·6	21·2	8
60 min	29·6	8·8	37·2	4

In these examples, each from one pair of frogs, 217AO had been injected intraventricularly at the time indicated prior to killing the frogs. Tissue homogenates were prepared from each segment of brain and nerve and 0·1 ml portions of the homogenates were preincubated with buffer-dye, diluent, and water. Also 0·1 ml samples of control tissue homogenates and a combination of 0·1 ml each of homogenates from control and inhibited segments were preincubated in the same way. After 30 min, the pH was adjusted to 8·6, and 0·1 ml of acetylcholine was added to each tube in a total volume of 1·2 ml for the usual assay. The calculation for correcting the inhibited samples was:

$$\% \text{ Inhibition} = \frac{E(\text{Control}) + E(\text{Inhibited}) - E(\text{Control} + \text{Inhibited})}{E(\text{Control})}$$

E.g. For the sample of spinal cord 6 hr after inhibitor administration:

$$\% \text{ Inhibition} = \frac{29{\cdot}6 + 2{\cdot}8 - 23{\cdot}2}{29{\cdot}6} \times 100 = 31\%$$

Reading from curve (1) Fig. 1, the inhibitor level causing 31% inhibition after 30 min preincubation is equivalent to that causing a 3% inhibition in assay without preincubation. Therefore, the value from the original assay (measuring hydrolysis at 0–10 minutes after homogenization of the tissue) was corrected by multiplying by $\frac{100}{97}$.

the combined sample (column 3). If the values in column 3 are smaller than the sum, this difference is to be ascribed to free inhibitor and may be expressed as percentage inhibition of the uninhibited samples (column 1) as shown in the table. Since acetylcholine has been added to the assay vessel after a 30 min pre-incubation in this experiment, in contrast to the assays for cholinesterase inhibited *in vivo* in which the substrate is added within 1 min after removal of the tissue and continued for 10 min, the inhibition after preincubation by the amount of free inhibitor would be read from curve 1, Fig. 1, but then the effect of this level of inhibitor on the assay would be read from curve 4, *e.g.*, 8 per cent of inhibition due to free inhibitor would have no effect in the assay without preincubation, while the highest inhibition found due to free inhibitor (38 per cent) would correspond to 7 per cent inhibition in the assay without preincubation.

TABLE 3.—THE EFFECT OF 217AO ON THE CHOLINESTERASES OF THE NERVOUS SYSTEM AND BLOOD (RANA PIPIENS)

	Brain	Spinal cord 1	Spinal cord 2	Plexus 1	Plexus 2	Sciatic nerve 1	Sciatic nerve 2	Blood
Length (mm)	—	7·5	9·6	8·9	8·9	12·8	12·6	—
ACETYLCHOLINE								
Normal activity	170 ± 9	162 ± 11	160 ± 9	76 ± 8	77 ± 7	82 ± 8	82 ± 7	22·4 ± 2·3
Time: 15 min	74 ± 10*	59 ± 5*	75 ± 7*	62 ± 9*	66 ± 7*	78 ± 7*	96 ± 10	81 ± 8*
30 min	37 ± 4*	40 ± 4*	28 ± 2*	57 ± 7	57 ± 6*	76 ± 8	88 ± 5	40 ± 15*
45 min	29 ± 3*	27 ± 2*	29 ± 2	42 ± 4*	46 ± 5*	52 ± 5*	84 ± 5	29 ± 8
60 min	24 ± 2*	23 ± 1*	22 ± 1*	37 ± 5	40 ± 1*	37 ± 1*	62 ± 4*	25 ± 5
75 min	22 ± 2	22 ± 4	23 ± 3	33 ± 1	35 ± 2*	32 ± 4*	38 ± 4*	17 ± 3*
BUTYRYLCHOLINE								
Normal activity	21 ± 5	37 ± 7	38 ± 6	51 ± 6	50 ± 2	56 ± 6	58 ± 9	10·8 ± 1·9
Time: 15 min	106 ± 10	93 ± 10	103 ± 9	87 ± 25	97 ± 10	88 ± 8	88 ± 11	81 ± 10*
30 min	89 ± 3*	99 ± 13	96 ± 10	83 ± 14	93 ± 9	88 ± 14	84 ± 10	44 ± 7*
45 min	74 ± 5*	53 ± 4*	76 ± 10*	66 ± 2*	64 ± 9*	68 ± 10	76 ± 7	40 ± 10
60 min	65 ± 4*	46 ± 8	59 ± 9	53 ± 3	61 ± 5	53 ± 6*	63 ± 6*	39 ± 4
75 min	59 ± 1*	50 ± 9	77 ± 16	47 ± 5	55 ± 6*	53 ± 4	57 ± 5*	39 ± 6
ACETYL β-METHYLCHOLINE								
Normal activity	80 ± 6	63 ± 6	65 ± 7	11·4 ± 2·1	8·7 ± 0·8	10·9 ± 0·9	9·7 ± 1·2	6·4 ± 2·5
Time: 15 min	54 ± 6*	47 ± 3*	48 ± 7*	73 ± 6*	72 ± 8*	83 ± 4*	104 ± 10	82 ± 8*
30 min	26 ± 2*	26 ± 3*	26 ± 1*	48 ± 6*	49 ± 6*	53 ± 18*	88 ± 18	52 ± 9*
60 min	20 ± 3*	18 ± 2*	22 ± 2*	34 ± 11	33 ± 6*	45 ± 4	52 ± 5*	27 ± 6*
75 min	16 ± 4	15 ± 3	19 ± 4	30 ± 1	34 ± 5	32 ± 2*	40 ± 8	20 ± 4

The inhibitor was injected into the fourth ventricle of the brain in a dose of 0·05 ml of 5×10^{-5} M-217AO/ frog. The normal activity is expressed as moles $\times 10^{9}$ acid released/mg protein/minute. All other values are percentages of the normal activities ± S.D. Values marked with an asterisk differ from the values immediately preceding them at the 1% level of significance.

The amount of *in vitro* inhibition was found to be negligible in all segments other than brain and the proximal segment of spinal cord after intraventricular injection of 217AO. In these segments, the maximal *in vitro* inhibition occurred at 60 min, and was no longer measurable after 24 hr. The quaternary 217MI, injected intraventricularly, remained in the central nervous system for a longer time, reaching a maximum at 6 hr, and remaining for 3 days in the proximal segment of the spinal cord. All enzyme assays of inhibitor-injected frog tissue were corrected for this *in vitro* inhibition whenever necessary.

RESULTS

When the penetrating inhibitor, 217AO, was introduced directly into the brain, a dose of 5×10^{-9} moles of inhibitor in 0·05 ml was maximally effective in inhibiting brain cholinesterase after 30 min (Fig. 4). However, half of this dose ($2{\cdot}5 \times 10^{-9}$ moles in 0·05 ml) was introduced routinely to decrease the effect of *in vitro* inhibition of brain and spinal cord samples. As the average frog weighed 25 g, this dose was equivalent to a dose of 10^{-7} moles drug/kg body weight.

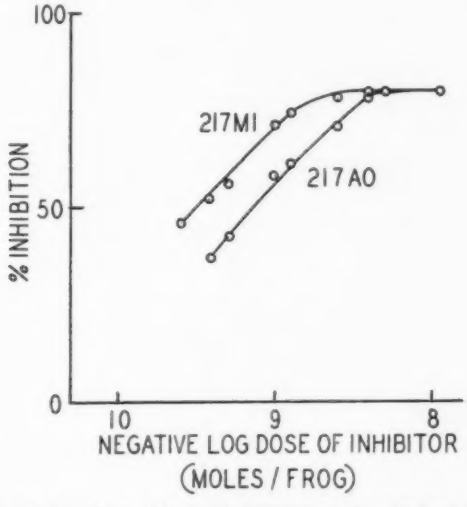

FIG. 4.—The *in vivo* inhibition of brain cholinesterase after the intraventricular injection of inhibitor.
Three frogs (av. wt. = 25 g) were killed 30 min after the injection of the dose of inhibitor in a volume of 0·05 ml for each point on the figure, and the brains assayed with acetylcholine as substrate.

Over 50 frogs were injected intraventricularly with this dose and killed in pairs at 15-min intervals after the injection, so that corresponding segments could be combined to obtain accurate values in animals with low enzyme levels. In these frogs a more concentrated homogenate was prepared so that each ml contained 2–3 mg of protein. The lowest enzyme activity, that versus acetyl β-methylcholine in nerve segments, was then 5–10 times the sensitivity of the method when a 0·2 ml portion of homogenate was assayed. At each point in time 3–5 groups of 2 frogs each were used to establish individual variation. Additional groups were assayed to measure the free inhibitor at each point and to rotate the order of assay so that blood, brain, and the proximal segment of spinal cord could each be assayed first after killing the frogs. The changes in the level of total cholinesterases and the true and pseudo enzymes in each segment as a function of time are given in Table 3. The control levels were derived from

nervous tissue samples of twenty-three frogs injected with 0·05 ml of frog Ringer solution and killed at the various times corresponding to that at which the drug-injected frogs were killed. The differences between one value and the one immediately preceding it which are significant at the 1 per cent level are marked with an asterisk.

The level of true cholinesterase in the central nervous system was never decreased to less than 15 per cent of the original level, even when a twenty-fold dose of the drug was injected intraventricularly. In peripheral nerve, about 30 per cent of the enzyme remained at the time of maximal inhibition of the true enzyme which occurred

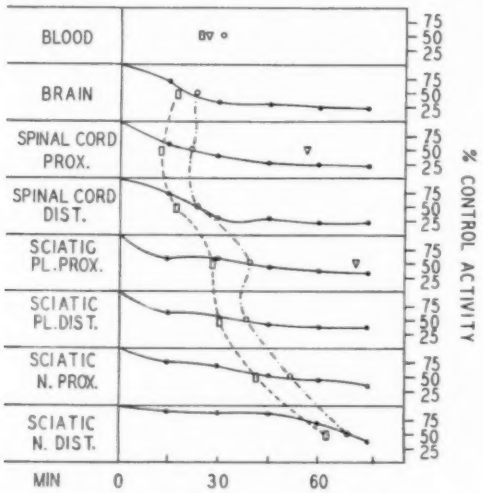

FIG. 5.—Time for 50% inhibition of cholinesterase after the intraventricular injection of 217AO.

The values for the hydrolysis of AcCh at 15–75 min after the injection of 217AO are plotted as percentage of control activity, and the times for 50% inhibition (open circles) in the nervous system are connected by a dot-dashed line. Superimposed are a dashed line marking the time for 50% inhibition of MeCh hydrolysis in each segment (rectangles) and triangles marking the time for 50% inhibition of BuCh hydrolysis whenever reached before 75 min. The 50% inhibition of cholinesterases in lysed whole blood is similarly shown.

6 hr after the injection. The level of the pseudo enzyme was eventually lowered to 50 per cent of the control level in brain and to 33 per cent of the control level in nerve (see succeeding paper).

The proximal segment of spinal cord was inhibited first, a not surprising finding in view of the proximity of the injection site, followed by the area on either side of this segment: brain and the distal segment of spinal cord, and then by nerve, all in a relatively short time. When the times of 50 per cent inhibition of total and true cholinesterases are plotted for each segment, the rate of penetration of the inhibitor may be calculated (Fig. 5). The rate of inhibition of pseudocholinsterase was much slower with only that in the upper spinal cord and plexus segments reaching 50 per cent inhibition in the first 75 min. True cholinesterase levels, as measured by the rate of hydrolysis of acetyl-β-methylcholine, showed a similar pattern to that of the total enzyme, but with an even more rapid rate of inhibition. On the basis of the values from which Fig. 5 was derived, and the average segment lengths recorded in Table 3, the rate of penetration of 217AO throughout the spinal cord and brain was 1·81 mm/min, through the plexus it was 1·26 mm/min, and the sciatic nerve 0·82 mm/min. The

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blood cholinesterases were also inhibited by 50 per cent in less than 30 min. However, some cholinesterase activity was found in each sample of blood assayed after the intraventricular administration of 217AO, and no free inhibitor could be demonstrated in blood, so that re-entry into the nervous system of excess inhibitor from the blood was unlikely.

The dose of the relatively 'non-penetrating' inhibitor, 217MI, which was necessary to achieve a substantial inhibition of brain cholinesterase, was obtained from the data in Fig. 4. A volume of 0·05 ml containing 10^{-9} moles of 217MI was introduced

TABLE 4.—THE EFFECT OF 217MI ON THE CHOLINESTERASES OF THE NERVOUS SYSTEM AND BLOOD

	Brain	Spinal cord 1	Spinal cord 2	Plexus 1	Plexus 2	Sciatic nerve 1	Sciatic nerve 2	Blood
ACETYLCHOLINE								
Time:								
60 min	35	41	48	95	96	98	98	75
2 hr	31	26	45	56	104	101	85	69
6 hr	31	27		60	94	104		69
1 day	25	30	41	70	89	96	87	71
3 days	21	25		85		107		104
BUTYRYLCHOLINE								
Time:								
60 min	74	74	95	87	89	106	108	93
2 hr	53	52	68	81	86	82	91	78
6 hr	51	57	65	99		84		87
1 day	59	78	57	88	90	90	88	81
3 days	100	84		83		85		107

The inhibitor was injected into the fourth ventricle of the brain in a dose of 0·05 ml of 2×10^{-5} M drug/frog. The values are percentages of the control levels of cholinesterase given in Table 3.

intraventricularly into 50 frogs. This dose corresponds to 0·4 of the dose of 217AO injected, since 217MI was 2·5 times more effective than 217AO when tested in either *in vitro* or *in vivo* systems. As the average frog weighed 25 g, the dose of 217MI was equivalent to a dose of 4×10^{-8} moles/kg body weight. With this inhibitor 4 frogs in groups of 2 each were used to establish each value. Additional assays at longer time periods were needed to establish the extent of inhibition. The changes in enzyme level may be seen in Table 4. In the central nervous system the rate of inhibition was much slower than with 217AP, but the same extent of inhibition was ultimately reached. The pseudo enzyme was also inhibited more slowly than with 217AO. The cholinesterases of the peripheral nervous system were not at all affected by 217MI introduced in this fashion, except for the true enzyme of the proximal segment of the plexus, which was consistently inhibited. Blood cholinesterases were only slightly inhibited.

After the intralymphatic injection of either drug the toxic symptoms were more severe than after an intraventricular injection of a dose equally effective in reducing the brain cholinesterase level. Death followed the intralymphatic injection of 5×10^{-8} moles of 217MI contained in 0·05 ml. A dose of 5×10^{-9} moles of either drug was

tolerated. Therefore, forty-eight frogs were injected with this dose, and the tissues assayed for cholinesterase activity. After the systemic injection of 217MI there was no inhibition in the central nervous system (Table 5) and slight inhibition in peripheral nerve, mainly of the pseudo enzyme in the sciatic nerve. The blood enzymes, however, were completely inhibited. After 217AO, there was slight general inhibition, particularly pronounced after several days in the central nervous system. The blood enzymes were again reduced to low activities.

TABLE 5.—THE EFFECT OF INTRALYMPHATIC INJECTION OF 217AO OR 217MI ON CHOLINESTERASES

	Brain		Spinal cord		Plexus		Sciatic nerve		Blood	
	217AO									
	Ac	Bu	Ac	Bu	Ac	Bu	Ac	Bu	Ac	Bu
Time:										
60 min	93	74	106	69	71	80	74	65	30	23
24 hr	83	74	107	64	81	74	68	53	13	24
2 days	87	88	70	87	65	74	67	63	49	64
3 days	68	119	72	88	83	82	88	70	87	89
	217MI									
	Ac	Bu	Ac	Bu	Ac	Bu	Ac	Bu	Ac	Bu
20 min	93	93	103	108	88	82	84	82	28	47
60 min	93	88	93	88	92	90	81	82	0	0
24 hr	107	84	110	108	84	54	78	48	0	0
2 days	98	94	97	93	80	60	73	45	49	35

The inhibitors were injected into the subcutaneous lymph sac in a dose of 0·05 ml of 0·1 mM drug/frog. The values are percentages of the control levels of cholinesterase given in Table 3.

DISCUSSION

The distribution of esterases in the nervous system of the frog is similar to that in other species. Although pseudocholinesterase has been found in sympathetic ganglia and the optic nerve of the frog (GIACOBINI, 1956; FRANCIS, 1953), the enzyme has not always been demonstrated in frog brain (SHEN *et al.*, 1955). In these studies, the level of the pseudo enzyme, as measured by the hydrolysis of butyrylcholine, was low in frog brain and spinal cord, but consistently present. In peripheral nerve it was the predominant enzyme hydrolysing acetycholine.

We have found that penetrating inhibitor 217AO inhibits the true cholinesterase of the sciatic plexus and nerve within 75 min after intraventricular administration to a level of 32–38 per cent of the control values while the pseudo esterase is inhibited to 47–57 per cent in the same time period. Both of these enzymes were inhibited progressively with time, although the true esterase showed a proximal-distal gradient of inhibition with time while the pseudocholinesterase inhibition seemed to progress uniformly in all segments. The speed of inhibition of the enzymes may find its explanation in the inhibitor leaving the brain and being circulated by the blood from which it diffuses to the different parts of the sciatic nerve. The progressive inhibition of the true enzyme in a proximal-distal direction would thus have to be related to the

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vascularization of the different parts of the plexus and nerve. It has been pointed out that after intracisternal injection no free inhibitor was found in the blood, a finding which makes it unlikely that the rate and pattern of inhibition may be explained by this mechanism. The vascular system as a route is further minimized by the longer time needed for inhibition in peripheral nerve after the intralymphatic injection of 217AO than after injection into the fourth ventricle. If the inhibition by free inhibitor from the blood is excluded, the time sequence of inhibition suggests a migration of the inhibitor within the nerve which may be either in the epi- or the endo-neural spaces or in the axoplasm itself. Transport by axoplasmic flow is least likely in consideration of the speed of movement of the inhibitor, since estimations of the rate of movement of molecules in the axoplasm are in the order of mm/day rather than mm/min. After the injection of [^{14}C]lysine into frogs a gradient of [^{14}C]lysine in the proteins of sciatic plexus and nerve was found (WAELSCH, 1958). If this gradient is taken as mirroring the flow of axoplasm, its rate would be in the neighbourhood of that estimated in mammalian nerve for axonal flow or rate of growth of generating nerve. It does not exclude that soluble small molecules migrate at a considerably faster rate. The rate of flow of ions in the endoneural spaces of the sciatic nerve of the guinea pig has been estimated as a few mm/hour by WEISS and his collaborators (1945) on the basis of the movement of ^{24}N and ^{64}Cu after injection between nerve fibres. This figure suggests that this or the epineural route is probably the main pathway for the rapid movement observed in our experiments, although the employment of all three routes appears possible before 217AO reaches the most peripheral sites of cholinesterase in sciatic nerve.

A flow of the inhibitor within the axoplasm would satisfactorily explain the lesser inhibition of pseudocholinesterase, since the pseudo enzyme has been localized in other species in the satellite cells of the nervous system, and particularly in the vascular endothelium, and the true enzyme at the neuronal surface and at the axonal terminations as well as in the cytoplasm of the neuron (KOELLE, 1951; BURGEN and CHIPMAN, 1952; and KOELLE and KOELLE, 1959). An alternative explanation may be that the lipophilic inhibitor flows in the endo- or epi-neural spaces with entrance into the axon only at the nodes of Ranvier, spreading from there within the axon. It appears reasonable that the inhibitor should have less difficulties penetrating the thin layer at the nodes of Ranvier than the layer of Schwann cells and myelin lipids at the internodal segments. Support for this suggestion may be seen in the fact that the lipophobic inhibitor administered systemically inhibits preferentially the pseudocholinesterase of the satellite cells.

The blood-brain barrier of the frog has been shown to have similar permeability properties to that of the rabbit as described by KOELLE and STEINER (1956); the lipophilic inhibitor readily transversed the barrier in either direction while the lipophobic inhibitor did not.

SUMMARY

The inhibitory action of lipid soluble and insoluble inhibitors on pseudo and true cholinesterases of the frog nervous system has been measured.

The rate of penetration of inhibitors to the sites of the enzyme in brain and peripheral nerve has been investigated after the introduction of the inhibitors either into the cerebrospinal fluid or into the blood. After introduction of the lipid soluble tertiary amine inhibitor 217AO into the fourth ventricle of the frog brain a proximal-distal progression of inhibition in time was found and a migration of the inhibitor of

approximately 1 mm/min through the sciatic nerve and plexus was estimated. The related ionic quaternary inhibitor, 217MI, similarly introduced, did not penetrate further than the most proximal segment of the sciatic plexus. The route by which the tertiary amine reaches the site of true cholinesterase in nerve is discussed.

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AMINO ACID AND PROTEIN METABOLISM OF THE BRAIN—VIII*

THE RECOVERY OF CHOLINESTERASE IN THE NERVOUS SYSTEM OF THE FROG AFTER INHIBITION

DORIS H. CLOUET and HEINRICH WAELSCH
New York State Psychiatric Institute and Columbia University College of Physicians and Surgeons, New York, N.Y.

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IN THIS PAPER the results of a study on the recovery of cholinesterases in the nervous system of the frog, after inhibition with a lipid soluble 'irreversible' inhibitor, are summarized. While there is no conclusive evidence that the reappearance of esteratic activity after inhibition should be considered protein synthesis, a study of the sequence of inhibition and recovery of the cholinesterases offers information which may form the basis for an understanding of the origin of these enzymic activities in the central nervous system and peripheral nerve. In the preceding communication the time sequence of inhibition of true and pseudocholinesterases in the central nervous system, sciatic plexus, and nerve, after administration of lipid soluble and lipid insoluble inhibitors, was described (CLOUET and WAELSCH, 1961). Within 75 min after the intraventricular administration of 217AO (obtained through the courtesy of Dr. R. A. LEHMAN of the Campbell Pharmaceutical Company of New York), acetylcholinesterase activity in the plexus and nerve was inhibited in rapid progression in a proximodistal direction. It was suggested tentatively from an analysis of the inhibition of the pseudo and true esterases that the inhibitor may travel within the epi- or endo-neural spaces along the plexus and the nerve and enter the axon at the nodes of Ranvier. This paper is concerned with the reappearance of enzymic activity after inhibition of the esterases by 217AO. In addition, data on the localization of the esterases in fractionated brain tissue from various animals are presented.

METHODS

Cholinesterase activity measurements. The methods of sampling of the frog tissues and of measuring esteratic activity with various substrates are described in the preceding report (CLOUET and WAELSCH, 1961). Briefly, the assay for cholinesterase consists of incubating a portion of brain or nerve

* Paper No. VII in the series is: CLOUET D. H. and WAELSCH H. The Penetration of Inhibitors into the Nervous System of the Frog (preceding paper).

A preliminary report of some of the material has been made in CLOUET D. H. and WAELSCH H. (1961) *The Regional Chemistry, Physiology and Pharmacology of the Nervous System.* Edited by KETY S. S. and ELKES J., Pergamon Press, New York.

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Abbreviations used: 2-diethoxyphosphinylthioethyldimethylamine acid oxalate, 217AO; reduced diphosphopyridine nucleotide, DPNH; 2-pyridine aldoxime methiodide, PAM; acetylcholine, AcCH; acetyl β-methylcholine, MeCh; butyrylcholine, BuCh; diacetylmonoxime, DAM; benzoylcholine, BzCh; mitochondria, Mt; microsomes, Mc.

TABLE 1.—THE LOCALIZATION OF CHOLINESTERASES IN FRACTIONS PREPARED BY CENTRIFUGATION

Tissue	Substrate	Homogenate	Nuclei	Mitochondria	Microsomes	Supernatant fluid
Frog—Brain	AcCh	165	54	210	433	5
	BzCh	21	8	12	18	0
	MeCh	68	6	92	182	0
Spinal cord	AcCh	141	51	177	406	2
	BzCh	35	42	20	31	0
	MeCh	60	13	84	170	0
Sciatic nerve	AcCh	70	50	87	175	0
	BzCh	54	52	48	104	0
	MeCh	9	8	12	22	0
Rat—Whole brain	AcCh	79	28	123	203	31
	BzCh	17	15	15	21	11
	MeCh	34	9	53	87	16
Caudate nucleus	AcCh	230	57	343	486	61
	BzCh	25	24	24	27	6
	MeCh	101	12	155	219	18
Hypothalamus and thalamus	AcCh	98	30	151	190	42
	BzCh	12	8	15	21	6
	MeCh	42	6	71	77	7
Frontal cortex	AcCh	60	20	86	165	23
	BzCh	10	8	8	12	7
	MeCh	31	5	33	60	9
Pons and Medulla	AcCh	61	29	108	121	18
	BzCh	16	9	21	14	7
	MeCh	26	12	42	49	9
Cerebellar cortex	AcCh	43	4	70	120	12
	BzCh	17	2	18	19	2
	MeCh	17	1	23	49	8
Monkey—Caudate nucleus	AcCh	277	183	346	710	5
	BuCh	14	1	29	24	4
	MeCh	94	30	189	330	0
Corpus callosum	AcCh	26	11	32	51	0
	BuCh	19	17	29	41	0
	MeCh	8	3	10	14	0

Each area was dissected out and homogenized in 0·25 M-sucrose in a 10% homogenate in a Potter-Elvejhem glass homogenizer with 0·1 mm clearance and centrifuged at 600 g for 10 min. The 'debris' was suspended in the same volume of sucrose, rehomogenized, and recentrifuged. The combined supernatant solutions were centrifuged at 1500 g for 10 min, 12,000 g for 15 min, and 100,000 g for 90 min in order to isolate fractions containing predominantly nuclei, mitochondria, and microsomes (see text). The various particulate fractions were homogenized in water to make a suspension of 1 mg protein/ml. The homogenate was diluted with water to the same protein concentration. The substrates were AcCh = 0·5 mM-acetylcholine, BzCh = mM-benzoylcholine, BuCh = mM-butyrylcholine and MeCh = mM-acetyl β-methylcholine. The values express specific esterase activity as moles $\times 10^9$ substrate hydrolysed/mg protein/min.

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homogenate containing 0·2 mg of protein with a substrate, and measuring colorimetrically the acid release with time.

Localization of cholinesterases in brain fractions. Whole brain and samples of nerve from monkey, cat, guinea pig, rat, and frog were homogenized in 0·25 M-sucrose, and the homogenates submitted to fractional centrifugation as described in the legend of Table 1, according to the method of HOGEBOOM and SCHNEIDER (1950). Other conditions of fractional centrifugation carried out to assess the homogeneity of the particulate matter are described in the individual experiments. The absence of mitochondria in the microsomal fraction was ascertained by measurements of DPNH-linked cytochrome *c* reductase activity and its antimycin A sensitivity as described by MAHLER (1955) by measuring the reduction of cytochrome *c* at 550 mμ, since the microsomal reductase was found to be completely insensitive to antimycin A (GIUDITTA and STRECKER, 1959).

Although some of the mitochondrial and microsomal preparation were found to be relatively homogenous when examined by a phase contrast microscope, the particulate fractions should be defined by the centrifugal force required to sediment them rather than the predominant particle expected in the fraction.

RESULTS

Localization of cholinesterases in fractions prepared by centrifugation

The relative cholinesterase activity for various substrates of whole brain and other parts of the nervous system of the frog, rat, and monkey is shown in Table 1. Since the separation of areas was made by gross dissection, areas such as the caudate nucleus were contaminated by surrounding tissue. Values for the same fractions of other areas of monkey brain and nerve and for nervous tissue in the guinea pig were very similar to those in Table 1. Since the mitochondrial and microsomal proteins were approximately 35 per cent and 12 per cent, respectively, of the total homogenate protein, more of the enzyme sedimented with the mitochondria, but the highest specific activity, particularly of the true cholinesterase, was in the microsomal fraction. As noted previously by ALDRIDGE and JOHNSON (1959), relatively more pseudocholinesterase than true cholinesterase was in the nuclear fraction.

Since these results did not exclude the possibility that fractions sedimenting intermediately between mitochondria and microsomes contained most of the esteratic activities, rat brain homogenates were fractionated at various centrifugal forces between that necessary to sediment all nuclei and that for sedimenting microsomes. The antimycin A sensitivity of DPNH-linked cytochrome *c* reductase was measured in each fractionation to detect mitochondrial contamination biochemically. A microsomal fraction free of antimycin A sensitive reductase, and with no mitochondria seen in a microscopic examination, could be prepared by sedimenting between 59,000 **g** for 6 min and 100,000 **g** for 60 min. This fraction contained cholinesterase of high specific activity. Likewise, a mitochondrial fraction was prepared by sedimenting between 3000 **g** for 10 min and 12,000 **g** for 10 min; in this fraction the reductase was completely inhibited by antimycin A, and the specific activity of cholinesterases was lower than in the other fraction, but it still represented a considerable part of the activity of the original homogenate (Table 2).

Restoration of cholinesterases in the nervous system of the frog

In Tables 3 and 4 are shown the levels of total and pseudocholinesterase during the recovery of the enzymes after inhibition by an intraventricular injection of a dose of 0·05 ml of a 5×10^{-5} M solution of 217AO in frog Ringer solution (2·5 μm-moles inhibitor/25 g frog). As discussed in the preceding report, the possibility of *in vitro* inhibition of the enzyme by the free inhibitor in the tissue during homogenization and

TABLE 2.—THE RELATIONSHIP BETWEEN THE CHOLINESTERASE CONTENT AND THE ANTIMYCIN A SENSITIVITY OF THE CYTOCHROME *c* REDUCTASE IN BRAIN FRACTIONS

Particles	Centrifugal force	Cholinesterase		Cytochrome *c* reductase		
		specific activity	Total units	control	+antimycin A	+alcohol
Mt + Mc	3,000–59,000 g	159	6360	0·106	0·046	0·110
Mc	59,000–100,000 g	226	5520	0·254	0·258	0·262
Mt	3,000–12,000 g	68	2040	0·210	0·0	0·190
Mt + Mc	12,000–100,000 g	181	8145	0·260	0·200	0·260

The brains of three rats were combined for each experiment. The cytochrome *c* reductase activity is expressed in the units defined by MAHLER (1955) which is the fraction of a 1·00 optical density change at 550 mμ/min at 22°/mg protein. The specific activity of cholinesterase is expressed as moles $\times 10^9$ of acetylcholine hydrolysed/mg protein/min. The total units equal the specific activity times the number of mg of protein in the fraction. Since antimycin A was added in a 95% ethanol solution to make a final concentration of 20 μg/mg protein, a control assay with alcohol alone was included.

TABLE 3.—THE RATE OF RESTORATION OF CHOLINESTERASES IN THE FROG NERVOUS SYSTEM AND BLOOD

	Brain	Spinal cord (SC)		Plexus (P)		Sciatic nerve (SN)		Blood
		1	2	1	2	1	2	
Length of segment (mm)	—	7·5	9·6	8·9	8·9	12·8	12·6	—
Protein (mg)	2·383	0·788	0·893	0·223	0·240	0·269	0·239	—
Control level	170 ± 9	162 ± 11	160 ± 9	76 ± 8	77 ± 7	82 ± 8	82 ± 7	22·4 ± 2·3
Time:								
60 min	24 ± 2	23 ± 1	22 ± 1	37 ± 5	40 ± 1	37 ± 1	62 ± 4	25 ± 4
6 hr	28 ± 2	27 ± 2	28 ± 3	30 ± 2	32 ± 2	28 ± 4	31 ± 4	18 ± 18
1 day	24 ± 3	27 ± 1	33 ± 3	33 ± 1	38 ± 5	35 ± 4	35 ± 4	39 ± 4
2 days	23 ± 1	24 ± 2	28 ± 4	40 ± 5	37 ± 2	33 ± 3	33 ± 4	63 ± 7
3 days	24 ± 0	30 ± 4	31 ± 2	42 ± 10	40 ± 8	45 ± 8	47 ± 3	93 ± 7
4 days	24 ± 3	33 ± 1	31 ± 4	48 ± 6	51 ± 4	57 ± 10	60 ± 8	89 ± 7
5 days	29 ± 1	30 ± 2	32 ± 1	50 ± 3	67 ± 2	70 ± 5	77 ± 8	90 ± 3
6 days	27 ± 3	33 ± 2	29 ± 2	74 ± 8	94 ± 12	74 ± 3	90 ± 9	88 ± 6
7 days	31 ± 1	46 ± 3	52 ± 3	89 ± 10	102	98 ± 5	108	13
8 days	42	50	42	91	90	98	98	86
9 days	44	41	53	92	89	95	98	95
10 days	52	44	48	98	104	96	98	89
12 days	65	45	46	98	94	115	92	96
17 days	85	65	65	117	108	115	98	105
21 days	86	99	78	95	96	95	93	90
30 days	101	101	98	96	97	96	102	93

Over 100 frogs were injected with the organophosphorus inhibitor as described in the text and killed at various times after the injection. For each time point at least 3 sets of 2 frogs each were used to establish the cholinesterase activity (with 0·5 mM-acetylcholine as substrate) with its standard deviation. Several other pairs of frogs were killed at the shorter time periods after inhibitor injection to measure *in vitro* inhibition. The control values were derived from homogenates of segments of the nervous tissue from 20 frogs injected with 0·05 ml of frog Ringer solution and killed 15–120 min later. The control level is expressed in units of activity = moles $\times 10^9$ acetic acid produced/mg protein/min. The other values are percentages of the control level, also with standard deviations whenever the sample number $n > 6$.

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incubation must be excluded. Therefore, the level of free inhibitor in the preparations was measured in separate assays. After 6 hr no free inhibitor could be found in this series of experiments. In the 60 min values, corrections for *in vitro* inhibition have been made (for methods see CLOUET and WAELSCH, 1961).

TABLE 4.—THE RATE OF RECOVERY OF PSEUDOCHOLINESTERASE IN THE FROG NERVOUS SYSTEM AND BLOOD

	Brain	Spinal cord (SC)		Plexus (P)		Sciatic nerve (SN)		Blood
		1	2	1	2	1	2	
Control level	21 ± 5	37 ± 7	38 ± 6	51 ± 6	50 ± 2	56 ± 6	58 ± 9	10.8 ± 1·9
Time:								
60 min	65 ± 5	46 ± 8	59 ± 9	53 ± 5	61 ± 5	63 ± 6	63 ± 8	39 ± 12
6 hr	65 ± 13	57 ± 9	52 ± 4	42 ± 3	39 ± 3	34 ± 5	34 ± 3	33 ± 7
1 day	50 ± 6	59 ± 4	67 ± 4	39 ± 3	39 ± 3	38 ± 5	41 ± 3	73 ± 27
2 days	73 ± 13	84 ± 16	88 ± 13	40 ± 3	38 ± 5	40 ± 8	40 ± 4	96 ± 1
3 days	94 ± 13	93 ± 9	74 ± 7	47 ± 7	43 ± 7	40 ± 6	41 ± 8	87 ± 14
4 days	89 ± 3	83 ± 8	74 ± 7	64 ± 8	69 ± 5	50 ± 5	50 ± 5	110 ± 27
5 days	96 ± 7	71 ± 9	70 ± 8	62 ± 11	69 ± 11	60 ± 2	50 ± 6	125 ± 9
6 days	86 ± 4	81 ± 7	88 ± 7	79 ± 4	95 ± 16	66 ± 4	67 ± 4	100 ± 16
7 days	106 ± 3	102 ± 10	116 ± 22	89 ± 12	105 ± 15	92 ± 9	86 ± 4	125 ± 35
8 days	97	86	96	99	94	98	98	143
9 days	102	95	99	98	95	94	94	142
10 days	135	125	124	94	107	93	98	225
12 days	112	97	107	87	92	107	105	189
17 days	84	92	95	96	107	91	93	116
21 days	91	94	86	94	102	96	94	100
30 days	96	132	107	96	98	97	99	137

The nervous tissue segments described in Table 3 were also assayed with 5 mM-butyrylcholine as substrate. In this table, the control level is expressed in units of pseudocholinesterase activity = moles × 10^9 butyric acid produced/mg protein/min. The other values are percentages of the control level with standard deviations.

In the central nervous system, the activity of the total cholinesterase did not increase significantly within the first six days after inhibition, and then increased rapidly until total recovery occurred between the 21st and 30th days. The pseudo enzyme recovered at a moderately regular rate in the central nervous system, completely recovering in 7 days.

In peripheral nerve, the total cholinesterase level was different for each segment of nerve. Recovery began in the first 24 hr, accelerating most rapidly in the distal segments (SN-1 and SN-2) at 2–3 days, in the P-2 segment at 3–5 days, and in the P-1 segment at 5–8 days. The level of pseudocholinesterase increased at a relatively constant rate, reaching the control level in 7 days. If the pseudocholinesterase values are subtracted from the total cholinesterase values, an estimate of the variation with time on true cholinesterase level is obtained (Table 5). The SN-2 segment recovered first, reaching the control value at 4–5 days and overshooting to 1·6-fold before decreasing. SN-1 reached the control value at 6–7 days, overshooting slightly. P-2 approached the control level at 7–8 days, and P-1 at the 10th day.

The quantitative aspects of the recovery may be seen in Table 6 where the total units of enzyme activity lost during inhibition are given in the top line, followed by the recovery of enzyme units at intervals of 5, 10, 17, 21, and 30 days. As shown in Tables 3 and 4, the total cholinesterase in nerve was restored completely in 10 days, while the total enzyme in the central nervous system was not restored completely until 30 days.

TABLE 5.—THE RECOVERY OF TRUE CHOLINESTERASE IN PERIPHERAL NERVE

	Sciatic plexus		Sciatic nerve	
	1	2	1	2
Control level	25	27	26	24
Time:				
1 day	9	6	7	5
2 days	10	9	5	4
3 days	9	10	15	14
4 days	3	4	19	20
5 days	6	16	23	34
6 days	17	25	24	35
7 days	14	27	28	38
8 days	18	23	25	24
9 days	21	22	25	25

When the pseudocholinesterase activity is subtracted from the total acetylcholine hydrolysis, the difference (in moles $\times 10^9$/mg protein/min) is a measure of true cholinesterase activity. The data are derived from Tables 3 and 4.

Reactivation of cholinesterases

Since the interpretation of the results depends on the degree of irreversibility of 217AO, several tests were applied to measure the ease of dissociation of the inhibited enzyme complexes *in vitro*. The reversibility of 217AO-cholinesterase inhibition *in vitro* as a function of time of storage was measured by injecting intraventricularly three frogs with a dose of 217AO(μm-mole/frog) which was effective in decreasing the brain cholinesterase content to 60 per cent of the control level, and three animals with frog Ringer via the same route. After 17 hr, at which time free inhibitor in the brain was no longer measurable, the animals were killed, and brain homogenates of each group were prepared. From these the mitochondrial-microsomal fraction was isolated by centrifugation, washed twice with sucrose, and suspended in water. This combined fraction contains most of the cholinesterase activity (see Table 1). The initial cholinesterase activity in each of the two preparations was measured, and then each was divided into three parts which were incubated with thymol as a preservative at 4°, 25°, or 37°. At intervals of 1, 2, 3, and 4 hr and 1, 2, 3, 4, 5, 9, 11, and 13 days, portions were removed from all incubations and assayed for cholinesterase activity and protein content (Fig. 1). Decreases in both protein level and cholinesterase activity were temperature-dependent, with the loss of enzyme activity proceeding at a faster rate than proteolysis. At 37°, all enzyme activity was lost in 3 days, and at 25° in 5 days. At 4°, 50 per cent of the specific activity of cholinesterases remained after 13 days. Even after 30 days at 4° a slight amount of activity was discernible in the control and

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in inhibited samples. There was an increase in enzyme specific activity during the first 3 hr at 25° and 37° and during the first two days at 4°. This increase was greater in the inhibited samples than in the control samples. However, since the absolute amount of acetylcholine hydrolysed per sample never exceeded the initial value, these differences reflect changes in the protein level due to proteolysis. The

TABLE 6.—THE QUANTITATIVE RECOVERY OF CHOLINESTERASES IN THE FROG NERVOUS SYSTEM

	Brain	Spinal cord		Plexus		Sciatic nerve	
		1	2	1	2	1	2
TOTAL CHOLINESTERASES							
Total units	404	128	143	17	19	22	20
Total loss	313·0	99·8	115·5	11·8	12·5	15·3	13·5
Recovery							
Day 1–5	4·0	8·3	7·2	4·1	6·3	9·5	9·9
Day 6–10	94·9	26·3	36·8	7·6	6·8	6·4	3·9
Sum	98·9	34·6	44·0	11·7	13·1	15·9	13·8
Day 11–17	85·1	27·0	29·3				
Day 18–21	48·7	15·4	30·0				
Day 22–30	65·0	20·8	22·9				
Sum	297·7	97·8	126·2				
BUTYRYLCHOLINESTERASE							
Total units	50	29	34	12	12	15	14
Total loss	25·0	15·0	16·3	8·1	8·5	10·5	8·9
Recovery							
Day 1–5	17·0	8·3	8·3	4·2	4·5	5·1	4·8
Day 6–10	6·8	6·6	8·3	3·5	3·7	4·5	4·0
Sum	23·8	14·9	16·6	7·7	8·2	9·6	8·8

The total units of enzyme are expressed as moles of acid released $\times 10^9$ per min for the segment. The units are the same for enzyme loss and recovery.

concentration of protein decreased in all samples, but to a slightly greater degree in the inhibited samples. A larger hydrolysis of acetylcholine in the inhibited samples would demonstrate reactivation. Since this was never found, there was no evidence of reactivation in these incubations. In another experiment, whole homogenates of frog brain were stored in the frozen state, then thawed and assayed at 10, 20, and 30 days. The enzyme activity in both control and inhibited samples decreased to about 80 per cent of the initial value during 30 days. However, the fraction of activity remaining in each sample was again the same.

An attempt was made to reactivate chemically *in vitro* the enzyme inhibited *in vivo*

by using PAM which is known to be a reactivator of organophosphorus-inhibited cholinesterase (WILSON and GINSBURG, 1955; HOBBIGER, 1956). PAM is an inhibitor of cholinesterase, causing 50 per cent inhibition of the hydrolysis of acetylcholine by frog brain homogenates in the present assay at a concentration of 1·0 mM which is in the range needed for reactivation. The usual assay for cholinesterase activity was, therefore, modified in an experiment in which an additional 30 frogs were injected intraventricularly with the usual dose of 217AO and killed in pairs at daily intervals 1–10 days after the injection. Tissue samples consisting of the brains (1), spinal cords

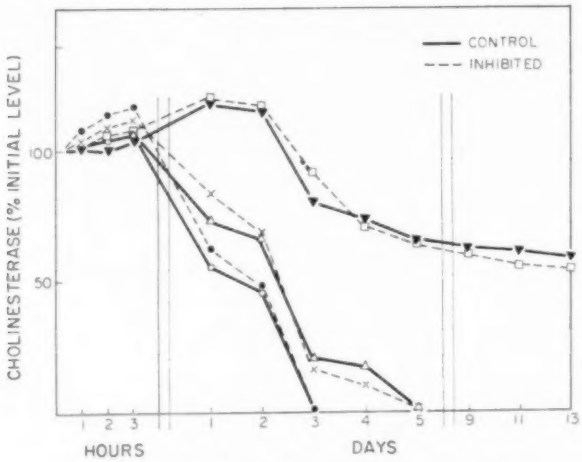

FIG. 1.—The loss of cholinesterase activity *in vitro*.

The mitrochondrial-microsomal fraction was prepared from brain homogenates of Ringer or inhibitor injected frogs as described in the text. The initial assay for cholinesterase in the samples from saline-injected frogs was 128 μm-moles of acid released/mg protein/min (= 100% in control series) and for the samples from inhibitor-injected frogs was 77 μm-moles of acid released/mg protein/min (= 100% on ordinate in inhibited series). The temperatures of incubation were: ○, ● = 37°; △, x = 25°; ▼, □ = 4°.

(2), and all segments of sciatic plexus and nerve (3) were homogenized in a four-fold more concentrated preparation, incubated at room temperature for 30 min in the presence of 1·0 mM-PAM, then diluted ten-fold by water before the addition of the assay constituents. Four incubations were run simultaneously for each tissue homogenate: tissue alone, tissue + PAM, tissue + AcCh, and tissue + AcCh + PAM, so that any inhibition due to PAM could be measured. The reactivatability of cholinesterase measured in this way is shown in Table 7. For each point one homogenate derived from two frogs was used; therefore, values of 10 per cent or less reactivatability are considered to be within the experimental error. For the first three days after the injection of 217AO the brain enzymes were reactivatable to the extent of 60–24 per cent of the inhibited enzyme, spinal cord cholinesterases to a lesser extent, and the nerve enzymes not at all. In experiments described in the preceding paper (CLOUET and WAELSCH, 1961), the level of free inhibitor in the nervous tissue segments, and the amount of *in vitro* inhibition due to the presence of this free inhibitor, was measured. Pertinent to the question of reactivation are two of the findings: (1) the *in vitro* inhibition in samples of spinal cord removed one day after the injection of 217AO amounted to 20 per cent of the total inhibition and (2) no free inhibitor was

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TABLE 7.—REACTIVATION OF CHOLINESTERASE WITH PAM

Days after 217 AO injection	Brain			Spinal cord			Sciatic nerve	
	Assay	+PAM	%	Assay	+PAM	%	Assay	+PAM
Control	165·2	163·0	(−2)	164·0	148·3	(−10)	88·8	88·8
Control	150·5	148·6	(−1)	132·0	137·5	4	75·3	79·6
1	26·0	105·2	60	18·9	68·5	38	28·4	28·4
1	21·6	96·5	55	22·6	62·0	31	26·2	28·0
2	29·5	84·6	43	23·1	69·5	37	23·8	21·8
2	21·8	97·0	55	23·2	52·5	24	26·7	26·7
3	45·9	81·2	32	23·0	48·2	20	27·6	30·0
3	50·2	76·5	24	28·6	35·9	6	35·8	34·6
4	59·8	71·5	12	32·0	40·0	7	37·7	37·7
5	66·4	65·0	(−1)	37·0	51·8	13	56·0	49·3
6	65·8	62·6	3	35·2	38·6	3	65·3	70·0
7	79·6	78·3	(−1)	40·2	41·2	1	77·7	78·0
8	79·4	84·0	7	55·5	49·3	(−5)	81·0	76·5
9	69·5	66·4	(−6)	52·5	51·3	(−2)	75·8	72·8
10	69·0	68·8	0	46·3	53·3	7	69·2	69·2

The tissue homogenates were incubated at room temperature for 30 min in the presence of mM-PAM, then diluted for cholinesterase assay. The results are expressed in enzyme units = moles × 10^9 acid released/mg protein/min. The % column gives the proportion of inhibited enzyme which is reactivatable.

found in peripheral nerve segments at any time. Thus, the amount of reactivation by PAM shown in Table 7 is the maximal value which could be expected *in vivo*.

Calculations of true cholinesterase levels in nerve

The values in Table 5, corresponding to differences between total and pseudocholinesterase levels, are rough indications of the changes in true cholinesterase activity in peripheral nerve. However, the actual level of true cholinesterase may be calculated if factors derived from the control levels are used in the calculation. In determining the normal level in nervous tissue homogenates of 20 frogs, acetyl β-methylcholine (MeCh) was used as a substrate to measure the activity of true cholinesterase as well as acetylcholine (AcCh) and butyrylcholine (BuCh) to measure total and pseudocholinesterase activity (see Table 3, preceding paper). However, the need to run a control without substrate as a measure of atmospheric acid in the laboratory air in this sensitive assay left only two cells for substrate in each set of four cuvettes in a spectrophotometric kinetic assay. Therefore, only AcCh and BuCh were examined as substrates subsequently and the true cholinesterase activity was calculated as described below.

Since all segments of the central and peripheral nervous system hydrolysed both MeCh and BuCh, one can calculate the approximate relative activity of each segment towards the three substrates by means of simultaneous equations:

$$x(\mathrm{Bu_1Ch}) + y(\mathrm{Me_1Ch}) = \mathrm{Ac_1Ch}$$
$$x(\mathrm{Bu_2Ch}) + y(\mathrm{Me_2Ch}) = \mathrm{Ac_2Ch}$$

in which x is the ratio of AcCh/BuCh for a sample and y is the ratio of AcCh/MeCh. From the assays of all segments of 20 control frogs, and of an additional 30 frogs which has been injected with 217AO, and in which all of the three substrates were

used, it was calculated that $x = 1{\cdot}12 \pm 0{\cdot}12$ and $y = 1{\cdot}86 \pm 0{\cdot}14$. Since the values of x and y were similar for each segment in control and injected frogs, the true cholinesterase activity in the various segments was obtained by subtracting 1·12 times the BuCh hydrolysis from the total AcCh hydrolysis. True cholinesterase levels calculated in this way are shown in Fig. 2 in which the ratio of true cholinesterase activity in the inhibited frogs to that in control frogs is plotted against time after the injection of 217AO for each segment of plexus and sciatic nerve. The time for maximal increase in

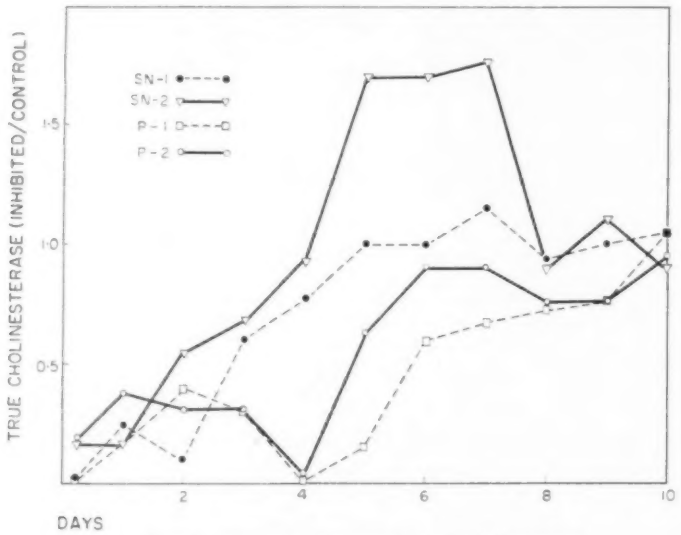

FIG. 2.—The rate of recovery of true cholinesterase.
The method of calculating these values is described in detail in the text. Proximal to distal order of the segments is: sciatic plexus (P-1, P-2) and sciatic nerve (SN-1, SN-2).

enzyme activity for each segment is more clearly seen as is the disto-proximal gradient of recovery. Two additional pieces of information have been obtained by this calculation: the extent of the 'overshoot' of true cholinesterase activity in SN-2 in 4–8 days, and the apparent initial small rise and fall of activity in the segments of the sciatic plexus in 1–4 days.

DISCUSSION

The activities of the cholinesterases found in homogenates of parts of the nervous system in rat, guinea pig, and monkey are comparable to other values in the literature (ORD and THOMPSON, 1952, ELKES and TODRICK, 1955). The content of true cholinesterase has been related to the number of neuronal elements, and that of pseudocholinesterase to the glial and vascular endothelial cells (HOLMSTEDT, 1959).

No pseudocholinesterase was detected in the toad brain by BRIGHTMAN and ALBERS (1959). However, with the present sensitive method enzymic splitting of both butyryl and benzoylcholine was found in frog brain, in agreement with previous findings on the occurrence of pseudocholinesterase in amphibian brain and spinal cord (GIACOBINI, 1959; FRANCIS, 1953).

The distribution of acetylcholinesterase in fractions obtained by centrifugation of homogenates in the caudate nucleus of the rabbit has been described by NATHAN and

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APRISON (1955) and in rat brain homogenate by TOSCHI (1958) and ALDRIDGE and JOHNSON (1959). As in these earlier studies, in the present study the cholinesterase activity of the nuclear fraction was always lower than that of the whole homogenate, and might be attributed to contamination of the fraction by unbroken cells. The supernatant fraction, too, contained only negligible cholinesterase activity. The mitochondrial fractions contained from 2·5 to 3·5 times more protein than the microsomal fractions, so that the bulk of the enzyme was always in the mitochondrial fraction, although a higher specific activity was found for the microsomal enzyme. The isolation of fairly separate fractions, quite distinct biochemically and by phase microscopy, which each contained large amounts of hydrolytic activity toward acetylcholine suggests either that the cholinesterase-containing component does not sediment in a single fraction or that there are two sites of enzyme localization. The same distribution of cholinesterase activity was found by ALDRIDGE and JOHNSON (1959) who have related the distribution of cholinesterases in fractions prepared by centrifugation of brain homogenates to that of succinic dehydrogenase, RNA, and esterase, and found that none of these components sedimented in the same way that cholinesterase did.

There have been indications that cholinesterase is localized in membranous structures. FUKUDA and KOELLE (1959) have found one site of cholinesterase activity on the membranes and surfaces of the neuronal cell, and an additional activity in the endoplasmic reticulum, and TOSCHI (1958) has succeeded in isolating microsomal cholinesterase by separating the brain microsomal particles into two fractions, one of which consisted of the membrane structures and to which all of the cholinesterase in the microsomal fractions was attached.

As to the mechanism of the reappearance of cholinesterase activity after inhibition, a number of possibilities present themselves: (a) reactivation of the enzyme, (b) resynthesis of the active sites, and (c) synthesis of the whole enzyme protein.

The reactivation of cholinesterase inhibited by organophosphorus anticholinesterese agents in *in vitro* systems has been accomplished in a number of ways, ranging from storage or dilution of the inhibited enzyme preparation to reactivation with hydroxamic acids and oximes. When inhibitor and enzyme are incubated *in vitro* and a reactivator such as PAM or MINA (monoisonitroso acetone) is added, complete reactivation of the cholinesterases can be obtained (CHILDS *et al.*, 1955; WILSON and GINSBURG. 1955). However, the reactivatible phosphorylated enzyme first formed is transformed into a form which cannot be reactivated after times ranging from 1·5 to 36 hr after the original inhibition (HOBBIGER, 1956; DAVIES and GREEN, 1956). In a study of a series of inhibitors and 2-(hydroxyiminomethyl)-*N*-methyl pyridinium methanesulphonate as the reactivator, BLABER and CREASY (1960) found that rat brain true cholinesterase was reactivatible from 1–3 days after inhibition depending on the stability of the inhibitor-enzyme complex. In *in vivo* inhibition, too, the lapse of time between the administration of the inhibitor and the reactivator determines the efficiency of reactivation, with almost simultaneous administration being necessary to decrease the mortality after LD_{100} doses of the inhibitor (LEHMAN *et al.*, 1960).

In our own experiments, cholinesterase which had been inhibited *in vivo*, showed no reactivation in *in vitro* incubations without added reactivator. Although some diethylphosphorylated cholinesterases of the type expected with 217AO have been reactivated by hydroxylamine (WILSON, 1951) and by DAM (CHILDS *et al.*, 1955), these reactivators were not effective in our system. Similarly, LEHMAN *et al.* (1960)

found that DAM was ineffective in reactivating cholinesterase inhibited *in vitro*, although PAM was a reactivator of which the effectiveness was related to the length of time that the inhibitor and enzyme were incubated together. However, reactivation by the addition of oximes would not be pertinent to the question of reactivation *in vivo*, unless one postulates the presence of a naturally occurring reactivator in frog tissue.

The lack of reactivation *in vitro*, and, particularly, the time lag of several days before the recovery of activity was apparent after inhibition, suggest that without the introduction of a hypothetical reactivator, the amount of reactivation occurring *in vivo* may be considered negligible. If one assumes that no reactivation did occur, rates of the synthesis of either the active site or of the whole enzyme molecule of pseudo and true cholinesterase in the frog nervous system may be calculated. The level of the pseudo enzyme was completely restored in 7 days, increasing at a rate of 12·8 enzyme units (15 per cent of the total loss) per day in the entire nervous system. The level of the true enzyme in the brain was almost completely restored at 30 days with a rate of 17·4 enzyme units (3 per cent of the total loss) per day. Comparable rates have been found in the rat brain by DAVISON (1955), who found over 50 per cent recovery of cholinesterase activity in 16 days, by OBERST and CHRISTENSEN (1956) who found a 56 per cent recovery 15 days after inhibition, and by BLABER and CREASEY (1960) who found that the recovery of true cholinesterase in rat brain occurred in an exponential fashion, reaching normal levels in about 5 weeks.

The sequence of enzyme recovery as related both to time and to anatomical localization is of greater interest than the time for total recovery of the cholinesterases. In peripheral nerve the recovery of the true cholinesterase was considerably more rapid than in the central nervous system, a relationship also found by KOENIG and KOELLE (1960) in the hypoglossal and cervical nerve trunks of the cat. The distal-proximal sequence of recovery of true cholinesterase in the sciatic plexus and nerve appears to contradict the hypothesis of the origin of axonal protein in the perikaryon and its transport within the axon by axoplasmic flow.

The evidence for the origin of the axonal content in the perikarya of the associated neurons derives from experiments in which bulging of a nerve was found proximal to a mechanical constriction. After the removal of the constricting cuff, the proximal bulge disappeared and the distal part of the nerve regained the diameter of the proximal segment (WEISS and HISCOE, 1948). The content of the enlargement of the nerve proximal to the constriction is indicated by the finding of the accumulation of enzymes (SAWYER and HOLLINSHEAD, 1945; HEBB and WAITES, 1956) and vesicles (VAN BREEMAN *et al.*, 1958) in the proximal end of a cut nerve. The experiments with cut nerves cannot be considered as decisive supporting evidence for axonal flow, but provide information as to the possible content of the dammed-up axoplasm in the constriction experiments.

After the administration of [^{32}P]phosphate, [^{14}C]lysine, or [^{35}S]methionine, the distribution of radioactivity with time was determined by SAMUELS *et al.* (1951), OCHS and BURGER (1958), WAELSCH (1958) and KOENIG (1958). In these experiments, carried out on the sciatic nerve of the guinea pig, frog, and cat, respectively, a proximal-distal gradient of ^{32}P radioactivity, as well as protein-bound amino acid, was observed. In unpublished experiments (WEISS, LAJTHA, and WAELSCH 1960, WEISS 1960) a gradient of radioactivity in radioautographs was observed in the nerves of chicken after administration of [^{14}C]glucose.

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All the experimental evidence quoted above is compatible with an origin of the axonal constituents by axonal flow from the cell body, although it cannot exclude their synthesis, possibly with involvement of satellite cells, stimulated at the place of constriction or the section of the nerve. The distal-proximal recovery of acetylcholinesterase reported in this paper as well as results of KOENIG and KOELLE (1960) appear to be incompatible with a mechanism of axonal flow.

Alternate mechanisms accounting for the appearance of cholinesterase and other proteins in the axon include axonal synthesis. It is not certain that the axon contains the apparatus needed for the synthesis of protein since the absence of ribonucleoprotein particles on the axoplasmic endoplasmic reticulum has been noted (VAN BREEMAN *et al.*, 1958). On the other hand, mitochondria have been shown to occur along the axon, being particularly numerous in the synapse. Experiments with muscle and liver mitochondria suggest that these cellular components are endowed with the ability to synthesize specific proteins. It could, therefore, be visualized that not the finished product but the cellular particle responsible for the synthesis of axoplasmic material is produced in the perikaryon and migrates through the axon to the synapse, some of the necessary substrates and coenzymes for the synthesis being supplied by the satellite cells or by epi- or endoneuronal or axonal flow. While the migration of the biosynthetic apparatus might satisfactorily explain the accumulation of enzymes in the distal end of a cut nerve, the development of a gradient along the nerve after the administration of [^{32}P]phosphate, [^{14}C]lysine, or [^{14}C]glucose would make necessary additional assumptions as to the dilution of these compounds either during migration or by local metabolic activity. Appearance of proximal-distal gradients and its reversal in time as in the experiments with [^{14}C]lysine represents the strongest biochemical evidence for the synthesis of axonal material in the perikaryon.

Another mechanism, involving the endoplasmic reticulum, may be suggested to explain the origin of axoplasmic proteins. Experiments with labelled amino acids have assigned to the endoplasmic reticulum which is sedimented as microsomes by fractional centrifugation a significant place in protein synthesis. While most of the experiments have been carried out with liver preparations, it has been also demonstrated *in vivo* and *in vitro* that brain microsomes show the highest rate of incorporation of labelled amino acid of all cellular elements (LAJTHA *et al.*, 1957; LAJTHA, 1960; CLOUET and RICHTER, 1959).

The endoplasmic reticulum of the perikaryon may by its own synthetic activity grow into the axon and its extensions may represent the force behind axonal flow. In the case of some proteins, the finished protein as part of the membranes of the reticulum may migrate down the axon. In other cases the proteins may obtain their final structure and activation during the growth of the reticulum by the metabolic activity of satellite cells or axonal mitochondria. Such reticular growth may result in the accumulation of large amounts of material at the nerve ending. Synaptic vesicles do accumulate at the nerve endings and proximal to nerve section. HEBB and SILVER (1961) have found that choline acetylase accumulated after section of the sciatic nerve of the goat in the proximal segment closest to the cut, and suggested that this accumulation is related to the accumulation of vesicles seen by VAN BREEMAN *et al.* (1958) since choline acetylase is found in synaptic vesicles. However, most of the membranes of the endoplasmic reticulum may disintegrate during their travel along the axon and the metabolic products of this disintegration may either furnish substrates for the

metabolism of the axon and synapse or diffuse out to the satellite cells. The rate of growth of the membranes by the biosynthetic activity of the endoplasmic reticulum within the cell body may under normal conditions, be balanced by the rate at which the membranes migrate along the axon. It is apparent that the results of the constriction experiments, of those in which the nerves were cut, and of those in which isotopic compounds were administered can easily be explained by assigning the endoplasmic reticulum a major role in the mechanism of axonal flow. This hypothesis may also explain the distal-proximal recovery after inhibition of acetylcholinesterase reported in this paper.

From histochemical evidence there are indications of two localizations of the cholinesterase in the nerve, one in an active form on the membranes and surfaces, and one as a newly generated form in the endoplasmic reticulum, separated in the microsomal fraction by centrifugation (FUKUDA and KOELLE, 1959). To fit the distal-proximal reappearance of acetylcholinesterase within the framework of axonal flow it could be visualized that the enzyme is synthesized and contained in the endoplasmic reticulum as this structure grows into the axon, and that the enzyme is either inactive or in amount too small to be noticeable as an increase along the axon. Accumulation or activation in distal-proximal direction would then result in the observations reported here. Recently LUBINSKA *et al.* (1961) have found that cholinesterase activity after transection of the phrenic nerve of the dog, shows a distal-proximal return in the portion of the nerve proximal to the cut. These observations, too, are explainable according to this hypothesis. However, information concerning a necessary corollary to this hypothesis is completely lacking at present. There is no experimental evidence suggesting a mechanism for the metabolic or mechanical disposal of the transported material either at the nerve ending or along the axon.

While the action of inhibitors of cholinesterases is measured by their specific effect on these enzymes it should be stressed that the action of inhibitors, particularly that of the organophosphorus inhibitors, extends beyond their inhibition of one group of enzymes. In this regard, a stimulation of the rate of incorporation of radioactive lysine into microsomal protein has been observed in experiments in which the turnover of the proteins of the caudate nucleus of the rat brain was measured with the aid of [^{14}C]lysine after inhibition of its cholinesterase by 217AO (CLOUET and WAELSCH, unpublished experiments). It was found that during the reappearance of the enzyme there is an increase of turnover rate of the microsomal protein greater than could possibly be accounted for by the resynthesis of cholinesterase. These results suggest that the inhibition of one enzyme may disturb total protein metabolism. However, 217AO inhibition may be a particular case, since this organophosphorus inhibitor attaches itself irreversibly to many tissue proteins. On the other hand, these experiments may indicate that the overproduction of enzyme during the recovery phase may find its reason in general disturbance of protein metabolism.

The study of the reappearance of the esterase after inhibition points to the complexity of the problem of the synthesis of specific proteins in the nervous system and suggests further approaches to this problem now under investigation.

SUMMARY

The sedimentation characteristics of the enzymes having hydrolytic activity against acetylcholine are the same for the fractions isolated by centrifugation from nervous

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tissue of the rat, guinea-pig, monkey, and frog. In each case, the highest total amount of activity was found in the mitochondrial fraction, and the highest enzyme specific activity in the microsomal fraction. When these two particulate fractions were separated by biochemical criteria, the cholinesterase activity could still be recovered in each of these fractions.

After irreversible inhibition of frog cholinesterases the original level of true cholinesterases was restored to the central nervous system in 30 days, and the pseudo enzyme in brain and nerve in 7 days. In peripheral nerve the restoration of true cholinesterase activity proceeded in a distal-proximal gradient. The concept of axonal flow in relation to these findings is discussed.

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THE BRAIN BARRIER SYSTEM—II

UPTAKE AND TRANSPORT OF AMINO ACIDS BY THE BRAIN*

ABEL LAJTHA and JENÖ TOTH
Department of Biochemistry, College of Physicians and Surgeons, Columbia University, New York, and the New York State Psychiatric Institute, New York, N.Y.

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THE UPTAKE and release of amino acids by the brain has only recently attracted the interest of the neurochemists. It has been shown that increase in concentration of most amino acids in the brain, after their elevation in plasma, is inhibited. The penetration into the brain of glutamic acid is strongly restricted (SCHWERIN, BESSMAN and WAELSCH, 1950), that of lysine (LAJTHA, 1958) and proline (DINGMAN and SPORN, 1959) fairly strongly restricted, that of glutamine (SCHWERIN *et al.*, 1950) and tyrosine (CHIRIGOS, GREENGARD and UDENFRIEND, 1960) not as much. The restriction is not absolute. Cerebral methionine, histidine, lysine and arginine were found to increase during continuous infusion (KAMIN and HANDLER, 1951). The restriction to uptake, measured with glutamic acid (HIMWICH, PETERSON and ALLEN, 1957) and lysine (LAJTHA, 1958), is not as strong in newborn as in adult animals. Apart from these data, very little is known about the mechanism and the rate of uptake and release under physiological conditions.

As part of a study of the mechanism of passage of metabolites into and from the brain, this paper reports changes in the concentrations of amino acids in the brain after their administration to the intact animal in various ways. It was found that part of the administered lysine and leucine leaves the adult brain against a concentration gradient of elevated plasma levels. Phenylalanine in the adult and leucine in the newborn brain did not show such behaviour under the experimental conditions employed.

EXPERIMENTAL PROCEDURE

C_3H mice and Sprague-Dawley (in experiments with leucine) or Sherman rats were used, young male rats, adult male mice, and newborn animals of both sexes. L-Leucine, L-lysine and L-phenylalanine were purchased from the California Corporation for Biochemical Research. For the intravenous administration mice were injected via the tail vein, rats under light anaesthesia via the leg vein with part of the skin opened; the site of subarachnoid injection (rat) was between the hemispheres about 2 mm below the dura at about the midpoint between the level of the eyes and the occipital protuberance. The animals were decapitated after the experimental period; whole blood or heparinized plasma and brain, liver and muscle were frozen in dry ice. To free brain of adhering blood and cerebrospinal fluid this organ was dipped in ice cold saline and blotted with filter paper before it was frozen. Free amino acids were extracted with 5% trichloroacetic acid (TCA), the TCA was subsequently removed with Dowex 2 (LAJTHA, FURST, GERSTEIN and WAELSCH, 1957), and the amino acid content was determined using a slight modification (LAJTHA *et al.*, 1957) of the method of HENDERSON and SNELL (1948). The blood content of several of the tissue samples was determined (GORDON and NURNBERGER, 1956) and all organ values were corrected for the amino acid content of the blood plasma present in the organ.

* For Part I see LAJTHA A. and MELA P. (1961) *J. Neurochem.* **7**, 210.

RESULTS

Cerebral amino acid efflux

In the first set of experiments the efflux of amino acids from the brain against a concentration gradient was investigated. Leucine was injected subarachnoidally into young rats to increase brain levels and at the same time plasma leucine levels were kept elevated by repeated intraperitoneal leucine administration. Frequent analyses of plasma content showed that this method of administration kept plasma levels well above those of the brain throughout the experiment, and infusion at a constant rate was not needed. Leucine levels in brain and plasma were measured at various times after administration (Table 1). Elevated cerebral free leucine concentrations decreased

TABLE 1.—EFFLUX OF LEUCINE FROM RAT BRAIN

Time (min)	Increase over control (μg leucine/g fresh tissue)					
	Lower plasma level			Higher plasma level		
	Plasma	Brain	Plasma / Brain	Plasma	Brain	Plasma / Brain
15	130 ± 40					
20	280 ± 20	110 ± 6	2·5			
25	160 ± 30					
30	180 ± 30	56 ± 10	3·2	590 ± 80	82 ± 12	7·2
40	230 ± 30					
45	250 ± 20	24 ± 6	10	980 ± 110	73 ± 8	13
50	250 ± 50					
60	300 ± 30	20 ± 5	15	770 ± 90		
85	190 ± 40					
90	260 ± 40	23 ± 3	11	710 ± 130	54 ± 10	13
150				870 ± 80	51 ± 5	17

Young male Sprague-Dawley rats (90–110 g) were injected subarachnoidally with 0·02 ml of 13 mg/ml leucine solution. Control leucine levels, plasma 19 ± 1, brain 15 ± 2 μg/g.

Lower plasma level: 10 min after the subarachnoid injection 0·6 ml of a 20 mg/ml leucine solution was injected intravenously, and 1 ml of this same solution intraperitoneally at 1 min then at 20, 30, 40, 50, 65 and 85 min.

Higher plasma level: 10 min after the subarachnoid injection 1·0 ml of a 20 mg/ml leucine solution was injected intravenously, and 1 ml of a 60 mg/ml leucine suspension was injected intraperitoneally at 1, 15, 30, 45, 60, 75, 100 and 130 min.

No animal was injected later than 5 min before the time of killing. One rat was used for each experiment; each time point is the average of three experiments.

even when plasma levels were kept above brain levels. Brain leucine levels decreased when plasma concentrations were kept at either of the two different levels, but they reached a lower level when plasma concentrations were kept lower. After about 45 min plasma to brain ratios reached a similar equilibrium value of 11–17 both when plasma levels were lower and when they were higher.

These experiments were repeated with lysine as the test amino acid. Under these conditions lysine also was transported out of the brain against a concentration gradient but to a smaller extent than leucine (Table 2). The decrease in lysine concentration took longer than the leucine decrease. Equilibrium seemed to be reached at a plasma to brain ratio of 5·6 with lysine, contrasted with about 13 with leucine. It is likely that the value of the plasma to brain ratio is not a constant for each amino

TABLE 2.—EFFLUX OF LYSINE FROM RAT BRAIN

Time (min)	Increase over control (μg/g fresh tissue)		Plasma / Brain
	Plasma	Brain	
30	520 ± 40	120 ± 14	4·3
60	480 ± 60	123 ± 11	3·9
115	470 ± 40		
120	500 ± 70	107 ± 18	4·7
150	530 ± 60	95 ± 13	5·6
160	510 ± 50		
180	360 ± 40	64 ± 12	5·6
240	340 ± 80	67 ± 13	5·1
360	410 ± 80	73 ± 19	5·6

Young male rats were injected subarachnoidally with 0·02 ml of a 14·6 mg/ml lysine solution in the beginning of the experiment, then intraperitoneally at 1, 40 and 70 min and every 45 min thereafter with 0·7 ml of a 90 mg/ml lysine solution; 0·12 ml of this same solution was injected intravenously at 15 min. Control lysine levels: plasma 65 ± 11, brain 49 ± 4 μg/g.

No animal was injected later than 5 min before the time of killing. One rat was used for each experiment; each time point is the average of three experiments.

TABLE 3.—EFFLUX OF PHENYLALANINE FROM RAT BRAIN

Time (min)	Increase over control (μg phenylalanine/g fresh tissue)					
	Lower plasma level			Higher plasma level		
	Plasma	Brain	Plasma / Brain	Plasma	Brain	Plasma / Brain
20	250 ± 20	140 ± 20	1·8			
45	200 ± 40	120 ± 10	1·7	2100 ± 400	280 ± 40	7·5
80	260 ± 50	130 ± 30	2·0	1900 ± 300	240 ± 30	7·9
120	180 ± 30	130 ± 20	1·4	2600 ± 300	310 ± 40	8·4
240				2000 ± 200	280 ± 30	7·1

Young male rats were injected subarachnoidally with 0·02 ml of a 16·5 mg/ml phenylalanine solution; and 15 min later intravenously with 0·5 ml of a 29 mg/ml solution. Lower plasma level: 1, 30, 60 and 90 min after the subarachnoid injection 1 ml of a 40 mg/ml phenylalanine suspension is injected intraperitoneally. Higher plasma level: 1, 35, 65, 100, 130, 170 and 220 min after the subarachnoid injection 1 ml of a 90 mg/ml phenylalanine suspension is injected intraperitoneally. Control phenylalanine levels: plasma 17 ± 3, brain 23 ± 1 μg/g.

One rat was used for each experiment; each time point is the average of three experiments.

acid, but is dependent on experimental conditions such as the absolute concentration of the amino acid in the plasma.

The experiments with phenylalanine (Table 3) showed no transport of this amino acid from the brain against a concentration gradient under similar experimental conditions. The plasma to brain ratio did not change with time. At plasma levels comparable to those reported in Tables 1 and 2, the value of the plasma to brain ratio with phenylalanine was less than 2, which was well below values of ratios obtained with lysine and leucine. The absolute brain level of phenylalanine was also dependent on the plasma concentration. Since the amino acid was administered into the spinal

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fluid levels, spinal fluid levels and consequently secondarily brain levels were influenced by the speed of exit of the amino acid from the spinal fluid into the blood. When plasma levels were higher, phenylalanine did not leave the spinal fluid as rapidly and thus penetrated brain tissue to a greater extent. In addition, the amino acid entered the brain directly from the blood.

Cerebral amino acid uptake

In order to determine if the lack of transport of phenylalanine from the brain is paralleled by an absence of any restriction to uptake by the brain, the effect of peripherally administered phenylalanine on brain levels was measured (Table 4).

TABLE 4.—PENETRATION OF PHENYLALANINE INTO RAT BRAIN

Tissue	μg phenylalanine/g fresh tissue		
	Control	Increase over control	
		20 min	45 min
Plasma	17 ± 3	310 ± 50	270 ± 60
Brain	23 ± 1	58 ± 6	67 ± 4
Liver	33 ± 1	180 ± 20	130 ± 30
Muscle	24 ± 2	250 ± 30	260 ± 50
Plasma/Brain		5·3	4·0

Young male rats were injected intraperitoneally with 1·0 ml of a 40 mg/ml suspension of phenylalanine in the beginning of the experiment and at 30 min (for the 45 min point). The animals were also injected at 15 min intravenously with 0·6 ml of a 29 mg/ml phenylalanine solution.

One rat was used for each experiment; each time point is the average of three experiments.

After an increase in plasma levels the brain levels were also increased, showing a net uptake. A restriction of uptake can be seen by comparing brain values to those in other organs; phenylalanine levels in liver and muscle were closer to plasma levels than were phenylalanine levels in brain. Little, if any, restriction was found for phenylalanine uptake by liver and muscle, but in brain the uptake was restricted. The plasma to brain ratio decreased during the experiment, showing continuing uptake by the brain; again it can be seen that the absolute value of this ratio was dependent on the absolute value of plasma levels.

The leucine content of mouse tissues was determined after its administration (Table 5). The respective injected doses were chosen in such a way that in the time interval studied (between 5 and 20 min after administration) the average plasma leucine level would be approximately equal in adult and newborn. Graphical calculation from concentration changes in blood in time showed a slightly higher average blood level in the adult than in newborn. Because of the intravenous injection in adults, versus the intraperitoneal administration in newborn, the adult brain was in contact in the first 5 min with blood of considerably higher leucine content than was newborn brain. Within the experimental period cerebral amino acid concentrations remained below that of plasma while values in liver were close to or higher than those in plasma.

In the beginning of the observation period, 5 min after leucine administration,

newborn and adult brain concentrations were similar; but at the end of the period, 20 min after leucine administration, newborn brain leucine levels were well above adult brain levels, in spite of the fact that in this time period the average plasma leucine levels were similar in the two age groups. Newborn brain concentrations increased while those in adults decreased with time. The decrease in brain level occurred in adult animals although the plasma level was several times that of the brain. The observation in adult mouse brain of the transport of leucine against a concentration gradient thus confirms the similar findings with rat (Table 1). Under these conditions leucine is not transported from newborn mouse brain against a concentration gradient.

TABLE 5.—PENETRATION OF LEUCINE INTO NEWBORN AND ADULT MOUSE BRAIN

Time (min)		Control μg/g	Increase over control μg/g			
			Low dose		High dose	
			5	20	5	20
Newborn	Blood	24 ± 3	63 ± 10	100 ± 25	670 ± 90	860 ± 120
	Brain	19 ± 2	16 ± 4	42 ± 8	58 ± 8	130 ± 18
	Liver	34 ± 3	180 ± 24	110 ± 12	740 ± 130	800 ± 95
Adult	Plasma	18 ± 3	130 ± 6	38 ± 9	910 ± 43	690 ± 72
	Brain	12 ± 2	16 ± 2	8 ± 3	64 ± 7	39 ± 8
	Liver	44 ± 7	120 ± 26	30 ± 7	1100 ± 140	690 ± 78

Low dose: newborn: 0·16 mg intraperitoneally (4 mg/ml solution); adult: 4·8 mg intravenously (20 mg/ml solution).

High dose: newborn: 0·8 mg intraperitoneally; adult: 16 mg intravenously and 16 mg intraperitoneally (20 mg/ml solution).

The average body weight of adults was 30 g, that of newborn 1·7 g. Three adult and 20 newborn C_3H mice were used for each experiment. Average of four experiments.

Calculated from Table 5, the average ratios of increase in leucine over control are the following: newborn blood/newborn brain = 6·2, adult plasma/adult brain = 11, newborn blood/newborn liver = 0·8, adult plasma/adult liver = 1·1. These ratios show the restriction to a net uptake of amino acids by the brain as compared to liver, this restriction being greater in adults than in newborn.

It should be noted that although in adults plasma was used for the determinations, in newborn this was not practicable and whole blood was used with no corrections made for this difference. A comparison of an amino acid level of whole blood with that of plasma after the intravenous administration of this amino acid into mice (Table 6) showed that the differences at 5 and 20 min in the case of leucine are small and cannot account for the differences in the brain amino acid levels at different ages.

In the experiments described (Table 5), adults were injected intravenously and newborns intraperitoneally. For further comparison, adult mice were injected intraperitoneally with leucine, and the leucine levels in plasma and brain were were measured at various times. The results (Table 7) supply further confirmation of the previously discussed experiments with leucine (Tables 1 and 5). The net increase in brain leucine content was again restricted. The plasma to brain ratio did

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not change greatly between 5 and 60 min. In these experiments, again as in the previous ones, there was a decrease in brain leucine levels at periods when the plasma levels were well above those of brain.

DISCUSSION

The present results, showing a decrease in adult brain concentrations against elevated plasma levels, point to the possibility of the participation of active transport processes in the efflux of amino acids from the brain. Several explanations, other than transport to the plasma from the brain against a concentration gradient, suggest themselves. Some of these will be discussed briefly.

TABLE 6.—AMINO ACID CONCENTRATIONS IN BLOOD AND PLASMA AFTER INTRAVENOUS INJECTIONS

Administered amino acid	Time (min)	(μg amino acid/g tissue)		Per cent difference
		Whole blood	Plasma	
Leucine	5	140	150	7
	20	31	34	9
Lysine	5	170	240	29
	20	103	121	15

Adult C_3H mice were injected intravenously with either 4·8 mg leucine (20 mg/ml solution) or 5·6 mg ysine (28 mg/ml solution).
Two animals were pooled for each experiment. Average of three experiments.

TABLE 7.—INTRAPERITONEAL LEUCINE ADMINISTRATION TO ADULT MICE

Time (min)	Increase over control (μg/g)		Ratio of increase Plasma/Brain
	Plasma	Brain	
5	450 $\pm$ 40	28 $\pm$ 5	16
20	700 $\pm$ 30	44 $\pm$ 4	16
45	440 $\pm$ 50	34 $\pm$ 2	13
60	370 $\pm$ 80	29 $\pm$ 4	13

Adult C_3H mice were injected intraperitoneally with 1·6 ml of a 20 mg/ml leucine solution.
Three animals were pooled for each experiment; average of three experiments.

1. The increment in the brain amino acids may be restricted to a small pool where the true brain concentration of amino acid is equal to that of plasma. In view of the plasma to brain ratios of 11–17 in the case of leucine, this pool would have to comprise less than 6 per cent of the brain with an 11–17 fold concentration as compared to the average free leucine level of the brain. (a) The excess amino acids could be restricted to the 'extracellular space', the size and existence of which is controversial at present. Against such an exclusion from the cellular spaces is the finding (Table 8, and unpublished) that if the administered amino acid was labelled, a significant portion of it was incorporated into cerebral protein. (b) The space to which the amino acid would be restricted could also be glial, and such a space would have to be postulated as of different sizes for lysine and leucine, with no such restriction for phenylalanine.

Although some kind of inhomogeneity in the free amino acid pools cannot be

ruled out—such pools in glutamic acid metabolism in the brain have been reported from this laboratory (LAJTHA, BERL and WAELSCH, 1960; BERL, LAJTHA and WAELSCH 1961)— the existence of small pools into which the net uptake of the amino acid is restricted does not seem to be the most likely explanation.

2. The amino acids may leave the brain, not through the blood but through the cerebrospinal fluid. This possibility does not dispense with transport against a concentration gradient, but would put the transport not between blood and brain but between brain and spinal fluid or spinal fluid and blood. Active transport of diodrast and phenolsulphonphthalein from the spinal fluid into blood has been shown recently (PAPPENHEIMER, HEISEY and JORDAN, 1961).

3. The amino acids may disappear not via transport but by cerebral metabolism. No significant amount of labelled metabolites of the three amino acids has been found in the brain after the administration of the uniformly labelled amino acids

TABLE 8.—EFFLUX OF LABEL FROM RAT BRAIN FOLLOWING [^{14}C] LEUCINE ADMINISTRATION

Time (min)	Counts/min/g fresh tissue		% of 5 min activity	
	Acid soluble	Protein	Acid soluble	Protein
1	2500	6	51	0
5	4800	57	99	1
20	1500	250	31	5
30	960	280	20	6
45	620	310	13	6
60	510	380	10	8

Young rats were injected subarachnoidally with 0·02 ml of a 13 mg/ml leucine solution (2 μmole and 13,000 counts/min to each animal).
One rat was used for each experiment; each time point is the average of three experiments.

under the experimental conditions described in this paper. The methods of separation and estimation and the results will be discussed in the next paper of this series (LAJTHA and TOTH, in preparation). Thus the metabolites would have to leave the brain at a great speed and almost completely.

That no hydroxy- or keto-analogues of lysine, leucine or phenylalanine were found in the brain in these short term experiments shows that under these conditions the microbiological method measures the level of amino acid in the brain rather than that of a metabolic derivative.

4. Amino acid levels could decrease by their utilization for net protein synthesis, an unlikely event in the non-growing adult brain. The fate of the injected label shows that this is not the case. Between 5 and 60 min after subarachnoid administration of uniformly labelled L-[^{14}C] leucine (Table 8) the acid soluble counts decreased by 89 per cent, but only 7 per cent of these were found in the protein fraction; this means that out of 4300 counts lost from the acid soluble fraction only 320 counts were incorporated into proteins.

While no previous results have indicated the participation of active processes in the exit of substances from the brain, recent results (CHIRIGOS, GREENGARD and UDENFRIEND, 1960) showing an inhibition of tyrosine uptake *in vivo* by some amino acids suggest the participation of active processes in the uptake of amino acids in

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living animals. Exchange diffusion or active processes are the most likely explanations for the findings of rapid exchange in spite of a barrier to net uptake with glutamic acid (SCHWERIN *et al.*, 1950; LAJTHA *et al.*, 1959) lysine (LAJTHA *et al.*, 1957; LAJTHA, 1958) and leucine (LAJTHA, 1959). In brain slices, active uptake dependent on metabolism has been shown for several substances, among them L-glutamic acid (STERN, EGGLESTON, HEMS and KREBS, 1949), D-glutamic acid (TAKAGAKI, HIRANO and NAGATA, 1959), 5-hydroxytryptophan (SCHANBERG and GIARMAN, 1960) and aspartic acid, but not glycine (KOREY and MITCHELL, 1951). Recently (NEAME, 1961), brain slices were shown to take up against a concentration gradient all of the six amino acids (histidine, proline, lysine, ornithine, methionine and arginine) that have been tried. The probable participation of active processes in the transport of metabolites to the brain *in vivo* has been generally recognized for some time, though experimental proof until recently was lacking. The transport of amino acids from the brain against a concentration gradient, and also the differences of exit rates of L-, as opposed to D-amino acids (LAJTHA, 1961), make it likely that these active processes play a role in the exit of substrates from the brain as well as in their entrance.

Active transport processes are not necessarily involved in the exit of all metabolites from the brain. Although leucine and lysine were transported out from the brain against a concentration gradient, the transport of phenylalanine under similar conditions was not demonstrated, suggesting perhaps a greater capacity of the brain for the net uptake of phenylalanine as compared to the other amino acids studied. The differences observed above make it likely that the level of each amino acid can be individually controlled.

Influx and efflux processes are governed by homeostatic control mechanisms, as shown by the ability of the brain to maintain under physiological circumstances higher levels of some of the amino acids than are present in the plasma, and also as shown by the restriction of a net increase in the cerebral amino acid levels after plasma levels have been elevated. Before any connection can be established between processes governing the uptake and processes governing the exit of amino acids from the brain, more of these compounds than the three in this paper will have to be studied.

The finding of a greater increase in leucine in newborn than in adult (Table 5), confirms similar previous findings with glutamic acid (HIMWICH *et al.*, 1957) and lysine (LAJTHA, 1958). Changes in brain permeability during development have been described with phosphate (BAKAY, 1953) and chloride (WAELSCH, 1955; LAJTHA, 1957) in addition to amino acids, though such changes have not been found with trypan blue (GRONTOFT, 1954; GRAZER and CLEMENTE, 1957). The mechanism of these changes is not fully understood at the present time and the changes are likely to be influenced concurrently by several factors.

The finding of transport of leucine against a concentration gradient from adult brain, although no such transport occurs under similar conditions from newborn brain (Table 5), gives another possible explanation for changes in brain permeability during development, namely, changes in transport, or rates of transport, out of the brain during development.

The results discussed in this paper make it clear that cerebral levels of compounds are determined by processes of efflux as well as influx. Changes in the efflux mechanism may also play a role in alteration of the brain barrier system by drugs, or during

development. The investigations reported here deal only with the brain, but similar considerations may apply to other organs as well.

SUMMARY

Transport of amino acids from brain against increased plasma levels has been studied after subarachnoid administration of leucine, lysine and phenylalanine to young rats and subsequent maintaining of increased plasma levels by repeated intraperitoneal injections.

Leucine was transported from rat brain against various elevated plasma levels and reached a plasma to brain ratio of about 11–17.

The transport of lysine against elevated plasma levels was slower than that of leucine and reached a lower plasma to brain ratio, about 5·6.

No transport of phenylalanine could be shown against elevated plasma levels.

Although no phenylalanine transport was found from the brain against a concentration gradient, the uptake of phenylalanine from the plasma by rat brain was restricted.

Following intravenous or intraperitoneal administration, the increased leucine content in adult mouse brain decreased in time against a concentration gradient of elevated plasma levels of this amino acid, to yield a plasma to brain ratio similar to that observed in rat at higher plasma levels.

The penetration of leucine into the brains of newborn and adult mice also was restricted. Elevated blood levels resulted in a greater net increase in newborn than in adult brain. The greater permeability to leucine in young brain thus parallels similar changes in the brain barrier system in respect to glutamic acid and lysine.

The participation of active processes in the transport of metabolites out of the brain and also the possible modification of these processes during development are discussed.

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CONCERNING INCORPORATION OF LABELLED CHOLESTEROL, FED TO THE MOTHERS, INTO BRAIN CHOLESTEROL OF 20-DAY-OLD SUCKLING RATS*

M. D. MORRIS and I. L. CHAIKOFF
Department of Physiology, University of California, Berkeley

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THE BIOSYNTHESIS of cholesterol in rat brain has been studied, with isotopic agents, by a number of investigators. WAELSCH, SPERRY and STOYANOFF (1940, 1941) administered deuterated water for four-day periods to several groups of baby rats between the day of birth and the 40th day of life. The mothers of some of these groups were also treated with heavy water. These workers reported that the younger the baby rat, the higher was the recovery of isotope from body fluids in the unsaponifiable fraction of its brain. By the 40th day the incorporation of ^{2}H into this fraction of baby brains was of the same low order as that observed in the mother's brain. It was concluded, that, in the early life of the rat, unsaponifiable lipids are synthesized in the brain. These findings were extended by SRERE *et al.* (1950). They measured the capacity of minced brains of new-born rats and of slices prepared from brains of adult rats to incorporate the ^{14}C of [1-^{14}C] acetate into cholesterol, and found that it resided only in the baby brains. They also noted that the percentage of the incubated [^{14}C] acetate recovered in cholesterol of the baby brain preparations surpassed that observed with liver, skin, and small intestine. The failure of injected acetate carbon to be incorporated into brain cholesterol of the intact adult rat has been reported by VAN BRUGGEN *et al.* (1952).

Although these investigations with intact rats and with surviving brain preparations appear to leave no doubt that cholesterol synthesis does occur in the baby rat's central nervous system, recent reports implying that cholesterol derived from plasma can be deposited in brain have appeared (DAVISON *et al.*, 1958, 1959). It would seem that the question whether cholesterol transported to the brain participates in the make-up of brain cholesterol has not been satisfactorily resolved. Data bearing on this point are presented here.

EXPERIMENTAL

Twenty-day-old, weanling Long-Evans rats, the mothers of which had been fed for 28 days (from 7 days prepartum to 20 days postpartum) a diet containing 0·05% of cholesterol labelled with [4-^{14}C] cholesterol, were killed by injections of sodium pentobarbitone. The diet (Table 1) was placed in pint Mason jars fitted with wire meshes to prevent access of the young to the food. At no time were the young rats observed eating this diet.

The livers and brains were rapidly removed and minced with a solution of 95% ethanol–ether (3:1), and the mixture so obtained was stored at -20° until analysed. The tissues were extracted once with 20 vol. of the above solvent and twice with 10 vol of ether. The combined lipid extracts were taken to dryness, and the residue was redissolved in hexane.

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Free cholesterol of liver* and brain† was separated by silicic acid chromatography by a modification of the methods of BARRON and HANAHAN (1958) and LA ROCHE (1957). The silicic acid was heated at 120° overnight and mixed with Johns Manville celite 545 in a ratio of two parts silicic acid to one part celite. A slurry of this mixture was added to jacketed chromatographic columns in hexane. The temperature of the column was kept constant by circulating tap water through the jacket. The column was washed with about 200 ml of hexane under 10 lb nitrogen pressure. The lipid extract dissolved in hexane was applied to the column, and the cholesterol esters and triglycerides were eluted with 150 ml each of 15% benzene in hexane and 5% ethyl in hexane, respectively. Free cholesterol was eluted from the column with 100 ml of 20% ethyl ether in hexane. In most cases this was sufficient to elute all of the free cholesterol, but in some cases absolute methanol was used to strip the column completely of free cholesterol.

TABLE 1.—COMPOSITION OF [4-^{14}C] CHOLESTEROL-CONTAINING DIET

Constituent	% of diet
Ground whole wheat	67·5
Casein (technical)	15·0
Skim milk powder	7·5
NaCl*	0·75
$CaCO_3$	1·5
Melted fat (hydrogenated vegetable oil)	6·7
Fish oil†	1·0
Labelled cholesterol	0·05

* Sufficient KI solution was added to the solid NaCl to furnish 0·9 μg I^- per g of diet.
† Contained 750 i.u. of Vitamin A and 300 i.u. of Vitamin D per g.

The eluate containing free cholesterol was taken to dryness under a stream of air, the residue was redissolved in ether, and the digitonides of a measured portion of the resulting solution were precipitated according to the method of SPERRY and WEBB (1951). The latter was washed, first with 4 ml as a 2:1 mixture of ether and acetone and twice with 4 ml of ether. After the final ether washing, the digitonides were dried at room temperature under a stream of air, and were dissolved in a measured volume of absolute methanol. Portions of the methanol solution were taken for colorimetric analysis (MORRIS *et al.*, 1957) of free cholesterol digitonides. Other 1 ml portions of this solution were added to 14 ml of toluene containing 45 mg of 2,5-diphenyloxazole, and the ^{14}C in this mixture was determined in a Packard tricarb liquid scintillation spectrometer. The efficiency of the ^{14}C counting was about 60 per cent.

The total cholesterol contents of new-born, 15-day-old, and 20-day-old rat brains were determined by the SPERRY-WEBB (1951) method. We are indebted to Mr. C. W. NICHOLS, Jr., for these measurements.

The [4-^{14}C] cholesterol was synthesized in this laboratory (MORRIS *et al.*, 1957). Just before its incorporation into the diet it was examined for purity by silicic acid chromatography and by the paper chromatographic method of KRITCHEVSKY and KIRK (1952) combined with the radioautographic procedure of KATZ and CHAIKOFF (1954).

RESULTS AND DISCUSSION

The brain of the one-day-old rat weighed about 240 mg and contained about 1 mg of cholesterol. The corresponding values for the 15-day-old rat were 1100 mg and 7·0 mg, and for the 20-day-old rat, 1200 mg and 9·6 mg. Thus, during the course of the experiment the baby rat brain acquired about 9 mg of cholesterol, practically all of which was in the free form.

* We have previously shown (MORRIS and CHAIKOFF, 1959) that the specific activities of free and esterified cholesterol in liver are equal.
† Essentially all cholesterol in the brain was in the free form.

The liver cholesterol of the 20-day-old rats had specific activities (Table 2) in the neighbourhood of 1000 counts/min per mg. Compared with these values, the specific activities of brain cholesterol (Table 2) of the seven 20-day-old rats were insignificant, and in three of them no ^{14}C activity was detected. At the time the 20-day-old rats were killed, blood and liver cholesterol were undoubtedly in isotropic equilibrium (MORRIS and CHAIKOFF, 1959) and we cannot, therefore, rule out the possibility that some of the ^{14}C activity in the brains of rats 2A, 2B, 6A, and 6B was derived from blood present in blood vessels of their brains. Even so, in the case of rat 6A, in which the highest value for the specific activity of brain cholesterol was observed, the actual counts, as determined by the method described above, were only twice that of the background. Thus,

TABLE 2.—SPECIFIC ACTIVITIES OF BRAIN AND LIVER CHOLESTEROL OF SUCKLING 20-DAY-OLD RATS, MOTHERS OF WHICH WERE FED A [4-^{14}C] CHOLESTEROL-CONTAINING DIET FROM 7 DAYS PREPARTUM TO 20 DAYS POSTPARTUM

Baby rat	Specific activity (counts per min per mg)	
	Brain cholesterol	Liver cholesterol
2A	26	
2B	7	
4B	0	840
5A	0	945
5B	0	920
6A	72	1125
6B	33	1350

our results provide evidence that dietary cholesterol—and for that matter, plasma cholesterol—contributes a negligible amount, if any, to brain cholesterol in the rat.

BLOCH, BOREK and RITTENBERG (1943) injected into an adult dog, daily for three days, an emulsion of deuterated cholesterol. Three days after the last injection they recovered labelled cholesterol in a large number of tissues, but not in the brain. More recently, however, DAVISON *et al.* (1958 and 1959) claim to have demonstrated the incorporation of radioactive cholesterol into the central nervous system of young rabbits and chickens that had received single injections of an emulsion containing [^{14}C]cholesterol. It is difficult to reconcile their conclusions with the findings presented here on the rat.

SUMMARY

Pregnant rats were fed a [^{14}C]cholesterol-containing diet for seven days before parturition, and the feeding of this diet was continued for the next 20 days during which they suckled their young. A study of the brains of the 20-day-old rats revealed either the absence of ^{14}C activity in brain cholesterol or recovery in that tissue of negligible amounts of [^{14}C]cholesterol. The conclusion is drawn that, in the rat, dietary cholesterol—and hence plasma cholesterol—contributes to a negligible extent, if at all, to the composition of brain cholesterol.

Acknowledgement—The technical assistance of Mr. WESTON KANE is gratefully acknowledged.

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NEUTRON ACTIVATION PAPER CHROMATOGRAPHIC ANALYSIS OF HUMAN CEREBROSPINAL FLUID*

ROLF BLOMSTRAND and FUMIO NAKAYAMA†
Swedish Medical Research Council, Unit for Biochemical Research on Atherosclerosis, University of Lund, Sweden.

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THE CEREBROSPINAL fluid is known to contain lipids, although in very minute amounts, 1 to 1·5 mg per 100 ml (TOURTELLOTTE *et al.*, 1958; BLOMSTRAND, 1960). It has been shown that the lipid composition of the cerebrospinal fluid may reflect certain neurological conditions in the brain and therefore might be used for diagnostic purposes (TOURTELLOTTE *et al.*, 1958). Because of the lack of suitable methods for the analysis of lipids on a microscale, lipids in cerebrospinal fluid have not been well characterized: however, using silicic acid column chromatography, BLOMSTRAND (1960) separated several lipid classes such as cholesterol esters, glycerides, free fatty acids and phospholipids from cerebrospinal fluid, and analysed the fatty acid composition of the phospholipids with the aid of gas-liquid chromatography. In recent work from this laboratory (BLOMSTRAND and NAKAYAMA, 1961), the combination of paper chromatography of phospholipids after mild hydrolysis according to the method of DAWSON (1960) with analysis by neutron activation proved very useful for the determination of phospholipids in human tissue when only minute amounts of sample were available. A similar neutron activation technique was used by STRICKLAND and BENSON (1960) for the determination of phosphate esters derived from phospholipids. In the present work this technique has been successfully applied to the analysis of the phospholipids in human cerebrospinal fluid.

EXPERIMENTAL

The analysis was carried out with 100 ml of pooled cerebrospinal fluid (CSF). Immediately after puncture, part of the CSF was taken for cell counting and the rest was centrifuged. Analysis showed that the cell count of the CSF and the protein content of the cell-free supernatant fluid were in the normal range. The cell-free CSF was concentrated under reduced pressure on a Mies filter (MIES, 1953), and extracted with chloroform–methanol mixture (2:1 v/v) followed by partition with water according to FOLCH *et al.* (1951) The chloroform layer was concentrated and the residue was subjected to silicic acid column chromatography to separate the phospholipids (BORGSTRÖM, 1952).

Chromatograms were prepared according to the method of REED *et al.* (1960) using filter paper impregnated with silicic acid and *n*-butyl ether–di*iso*butyl ketone–acetic acid–water (20:20:20:3, by vol.) as solvent. This was followed by neutron activation analysis.

The paper chromatography technique of deacylated phospholipid hydrolysate (BLOMSTRAND and NAKAYAMA, 1961) was essentially based on the method of DAWSON (1960). After alkaline hydrolysis of the phospholipids at 37° for 20 min (approximately 1 mg of phospholipids was obtained from 100 ml of pooled CSF), the aqueous layer containing alkali labile phospholipid hydrolysate was separated.

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† Present address: Department of Surgery I, Kyushu University Faculty of Medicine, Fukuoka, Japan.

The lower chloroform–*iso*butanol layer was washed twice with a little water and the washings were combined with the aqueous layer previously separated. The combined extract was concentrated under reduced pressure, and the residue was dissolved in 80% ethanol and applied to Whatman No. 1 paper for subsequent paper chromatography using phenol saturated with water, acetic acid and ethanol (100:10:12, by vol.). The chloroform–*iso*butanol layer was hydrolysed at 37° with 10% trichloroacetic acid for 30 min with vigorous shaking. After hydrolysis, petroleum ether was added, and the resultant aqueous layer containing alkali-stable acid-labile phospholipid hydrolysate was concentrated and applied to Whatman No. 1 paper for chromatographic analysis. The organic solvent layer containing alkali and acid stable phospholipids was hydrolysed with methanolic hydrochloric acid at 100° and the hydrolysate was applied to the paper for chromatographic analysis.

For neutron activation analysis, the paper chromatogram was rolled and sealed in an aluminum tube. After irradiation with a slow neutron stream (5×10^{11} neutrons/cm^2/sec) for 15 hr (irradiation was carried out at the research reactor at Atom Energi AB, Stockholm), autoradiograms were prepared using Kodak standard X-ray film (BLOMSTRAND and NAKAYAMA, 1961).

RESULTS

The autoradiograms prepared after neutron activation of one-dimensional paper chromatograms of alkali labile phospholipid hydrolysates from two samples of human cerebrospinal fluid are shown in Fig. 1 in comparison with those of human brain and rat liver. Spots were identified as GPC(glycerylphosphorylcholine; derived from lecithin), GPE(glycerylphosphorylethanolamine; from phosphatidylethanolamine), cyc-GP(cyclic-glycerophosphate; derived from lecithin and phosphoinositide), GPS (glycerylphosphorylserine; from phosphatidylserine), and GPI(glycerylphosphorylinositol; from phosphoinositide). A chromatogram of the alkali labile phospholipid hydrolysate of the human brain was run in a second direction and the neutron radiogram is presented in Fig. 2. It shows the better separation of each component, namely, of glycerylphosphorylserine (GPS) and glycerylphosphorylinositol (GPI). Lack of material prevented us from carrying out two-dimensional paper chromatography of cerebrospinal fluid phospholipids.

Glycerylphosphorylcholine derived from choline plasmalogen was identified in the autoradiogram of alkali-stable, acid-labile phospholipid hydrolysates of cerebrospinal fluid as well as of human brain and rat liver (Fig. 3). Plasmalogen is a phosphoglyceride which contains fatty acid residue, an aldehydogenic unit (vinyl ether), and glycerylphosphorylcholine or ethanolamine (HANAHAN, 1960).

The autoradiogram of alkali and acid stable phospholipid hydrolysate (Fig. 4) shows the presence of phosphorylcholine + sphingosylphosphorylcholine which arise from sphingomyelin in cerebrospinal fluid. There are also several unidentified spots. To determine whether or not lysolecithin was present, phospholipids of cerebrospinal fluid, human brain, and rat liver were subjected to silicic acid impregnated paper chromatography followed by neutron activation. Although paper chromatography after mild hydrolysis clearly indicated the presence of phosphatidylethanolamine, phosphatidylserine, phosphoinositide and choline plasmalogen in cerebrospinal fluid, the autoradiogram of the silicic acid impregnated paper chromatogram of cerebrospinal fluid phospholipids showed only three spots, i.e., lysolecithin, sphingomyelin, and lecithin. This type of paper chromatography requires a larger sample of material but differentiation of the components is no clearer.

The results presented indicate the presence of lecithin, lysolecithin, phosphatidylethanolamine, phosphoinositide, phosphatidylserine, choline plasmalogen, and sphingomyelin in human cerebrospinal fluid and brain.

DISCUSSION

Human cerebrospinal fluid has been shown to contain phospholipids in the range of 0·4–0·6 mg per cent (TOURTELLOTTE, 1958). Since 75 times more inorganic phosphorus is present than phospholipid phosphorus in cerebrospinal fluid, the lipid extracts can easily be contaminated with inorganic phosphorus to give too high phospholipid values. The use of FOLCH's (1951) extraction procedure followed by purification on silicic acid column chromatography would greatly diminish the possibility of contamination with inorganic phosphorus. Recently TOURTELLOTTE (1958) has made a considerable contribution to our knowledge of lipids in cerebrospinal fluid. He applied classical extraction techniques in combination with chemical analysis on a microscale to determine phospholipids in cerebrospinal fluid. However, in view of the extremely small amount of material generally available, the criteria and methods used by him cannot always be applied.

To our knowledge, paper chromatography has not been used before for analysis of phospholipids in human cerebrospinal fluid although as a microanalytical tool the technique is well established. However, in the case of phospholipids, the lack of suitable methods of detection limits the resolving power of paper chromatography. By combining paper chromatography of phospholipids after mild hydrolysis with subsequent analysis by neutron activation (BLOMSTRAND and NAKAYAMA, 1961), we were able to identify the phospholipids in cerebrospinal fluid on a microscale. The major phospholipids found were lecithin, phosphatidylethanolamine, and sphingomyelin. Lysolecithin, phosphoinositide, phosphatidylserine and choline plasmalogen were also present in small amounts. NIXON (1953) showed that inositol was present in cerebrospinal fluid in relatively high concentrations (2·7 mg per cent), but only a small part of this was in a combined form. The present investigation has shown clearly that part of the combined inositol is in the form of phosphoinositide. In an extensive work, STAMMLER (1954) showed that a considerable amount of plasmalogen (0·3 mg per cent) was present in human cerebrospinal fluid. Our results confirm and extend his result (Fig. 3). In order to get reasonably distinct autoradiograms, about 100 ml of cerebrospinal fluid were required in the present study. However, using higher neutron flux from a reactor now available, it would be possible to reduce the amount required to 25 ml, thus making it feasible to introduce this technique to the clinic for the investigation of cerebrospinal fluid. Studies on the fatty acid composition of plasma and cerebrospinal fluid reveal significant differences (BLOMSTRAND, 1960; BLOMSTRAND, DENCKER and SWAHN, 1960). These differences may reflect the difference in phospholipid composition in cerebrospinal fluid and plasma, which requires further investigation.

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SUMMARY

A microtechnique for the determination of the phospholipid components in cerebrospinal fluid is described in which paper chromatography after mild hydrolysis is followed by neutron activation. The method enabled us to analyse phospholipids with considerable reliability.

The major phospholipids identified were lecithin, phosphotidylethanolamine, and sphingomyelin but lysolecithin, phosphoinositide, phosphatidylserine, and choline plasmalogen were also present in small amounts. The possibility of introducing this technique for clinical use is suggested.

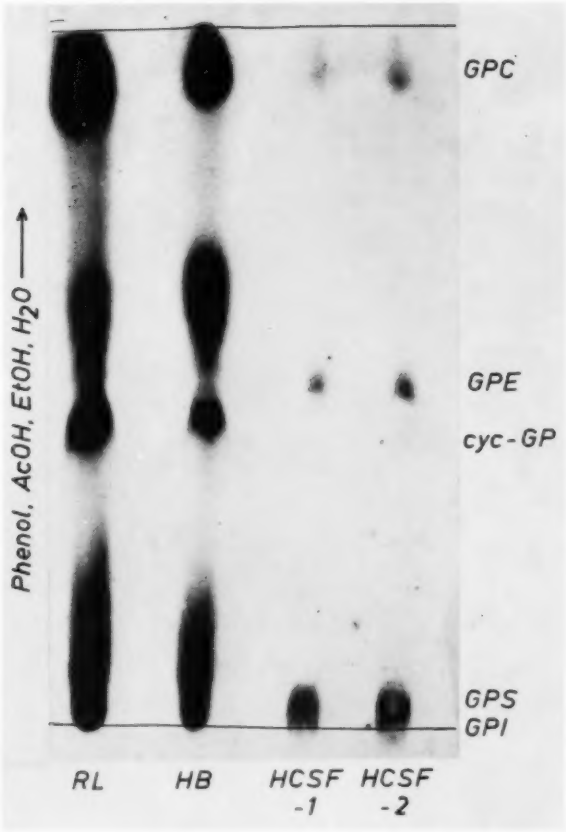

FIG. 1.—Neutron radiogram of one-dimensional paper chromatogram of alkali labile phospholipid hydrolysate of human cerebrospinal fluid (HCSF) in comparison with those of rat liver (RL) and human brain (HB). Chromatogram was run with phenol saturated with water, acetic acid and ethanol (100:10:12, by vol).

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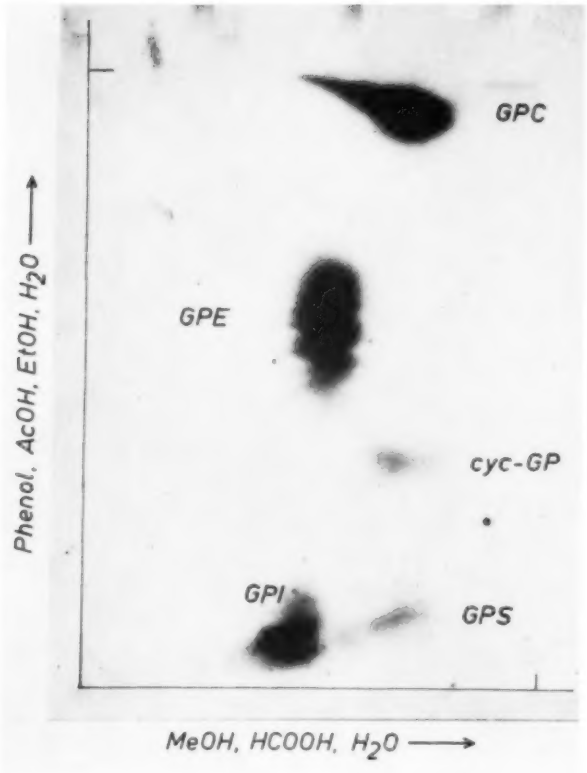

FIG. 2.—Neutron radiogram of two-dimensional paper chromatogram of alkali labile phospholipid hydrolysate of human brain. Chromatogram was run in one direction with phenol saturated with water, acetic acid and ethanol (100:10:12 by vol) and in a second direction with methanol–98% formic acid–water (80:13:7 by vol).

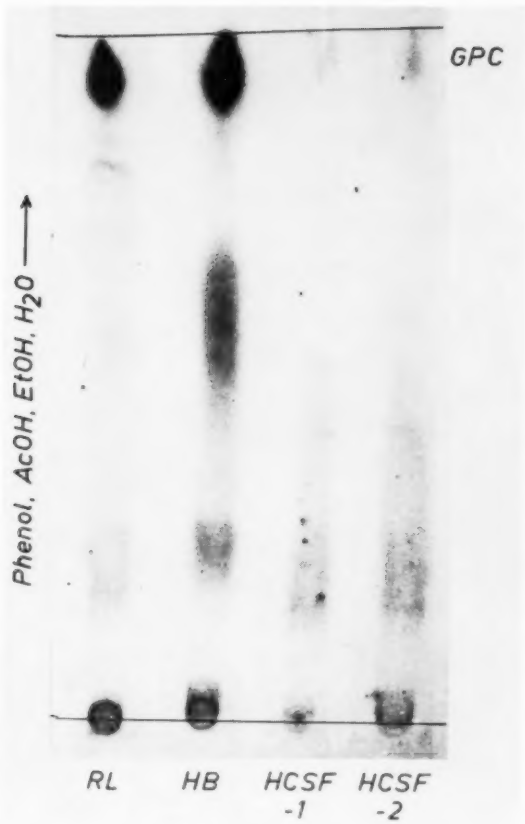

FIG. 3.—Neutron radiogram of alkali stable acid labile phospholipid hydrolysate of human cerebrospinal fluid (HCSF), rat liver (RL) and human brain (HB).

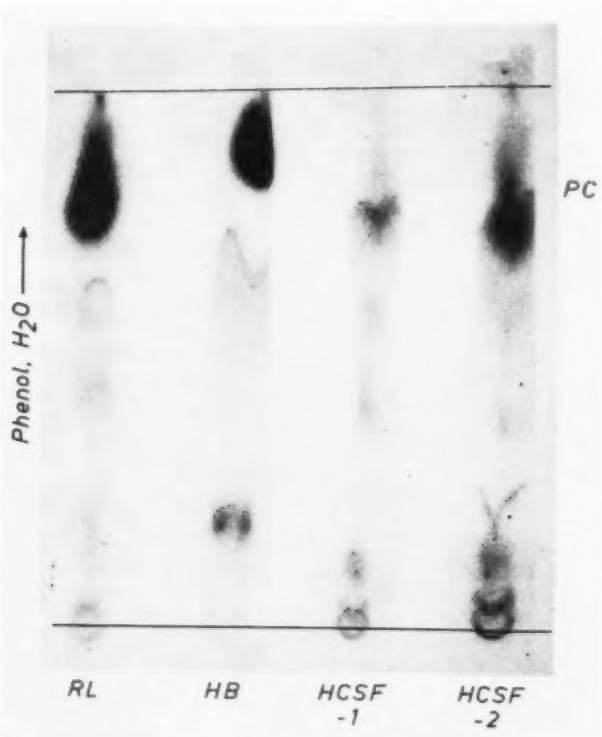

FIG. 4.—Neutron radiogram of alkali acid stable phospholipid-hydrolysate of human cerebrospinal fluid (HCSF), rat liver (RL) and human brain (HB).

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THE COMPOSITION OF BRAIN LIPOPROTEIN FRACTIONS OBTAINED BY DIFFERENT PROCEDURES*

M. K. GAITONDE

McLean Hospital Research Laboratories, Belmont, Massachusetts; Department of Neurology and Psychiatry, Harvard Medical School, Boston, Massachusetts; and Neuropsychiatric Research Unit, (M.R.C.), Woodmansterne Road, Carshalton, Surrey, England

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PRECIPITATION with trichloroacetic acid (TCA) followed by extraction of the precipitate with lipid solvents has been widely used as a method of tissue fractionation in biochemical investigations. The lipid extracts prepared in this way contain a certain amount of protein which has generally been assumed to consist of proteolipids; but the precise nature of this lipoprotein fraction has received little attention. Evidence has recently been obtained that the turnover of the proteins extracted by lipid solvents from the TCA precipitate of brain tissue is higher than that of the proteolipids prepared by the method of FOLCH and LEES (1951) by extracting fresh brain tissue with chloroform–methanol (2:1, v/v) (GAITONDE, 1961). The higher turnover of the protein fraction extracted from the TCA precipitate might be due to the presence of other lipoproteins in these extracts; or it might be due to phosphatido-peptides, which were obtained by FOLCH and LEBARON (1953) by re-extracting the trypsin-resistant protein residue under acid conditions with chloroform–methanol–HCl (200:100:1, by vol).

In the present investigation the lipoprotein fractions obtained by different procedures have been further characterized by determining their nitrogen, phosphorus and amino acid composition. It has been shown that the proteins extracted from the TCA precipitate differ in amino acid composition from the proteolipids prepared by the method of FOLCH and LEES. Fractions obtained by re-extracting the protein residues under acid conditions with chloroform–methanol–HCl (200:100:1, by vol). are described in the present paper as 'phosphatido-peptide fractions' since this fraction prepared under the conditions of FOLCH and LEBARON was shown to contain phosphatido-peptides.

MATERIALS AND METHODS

Reagents

All reagents were of analytical grade. *Phenol:* This reagent, without a preservative, was freshly distilled before use. n-*Butanol:* Traces of peroxides were removed by keeping butanol in contact with ferrous sulphate for 24–48 hr. The clear supernatant butanol was decanted, refluxed for 2 hr with reduced iron and distilled. It was stored in a dark bottle and used within 2–4 weeks. *Chloroform, methanol and acetone:* These solvents were redistilled before use. *Citrate buffer:* A solution of citric acid (21 g) in 200 ml water and 200 ml N–NaOH was brought to pH 5·0 (MOORE and STEIN, 1948) or to pH 7·5 with 2 N-NaOH as required. The volume was made up to 500 ml and the solution stored at 4°. *Ninhydrin spray reagent:* To a solution of ninhydrin (0·5 g) in 12·5 ml methanol was added a solution of $SnCl_2 \cdot 2H_2O$ (20 mg) in 6·25 ml of citrate buffer (pH 7·5). The contents were mixed gently and

* This work has been supported by a grant from the Ford Foundation for the Study of the Dynamic Equilibrium of the Structural Components of Brain.

made up to 100 ml with methanol. The reagent was prepared freshly before use. *Amino acids:* All amino acids were obtained from the California Foundation except taurine which was obtained from K & K Laboratories (New York). L-*Allo*-cystathionine was kindly provided by Dr. M. ARMSTRONG of the Fels Research Institute. N-Acetyl-L-aspartic acid was a gift from Dr. H. H. TALLAN of the Rockefeller Institute for Medical Research. Ethanolamine (Eastman Kodak) was redistilled before use. Each amino acid (20–25 mg) was weighed accurately, dissolved in 1 ml of 2 N-HCl, and made up to 25 ml with water. In routine work for quantitative paper chromatography of tissue samples the following two standard solutions of amino acids were prepared: Solution I contained glycine, arginine, lysine, phenylalanine and ethanolamine and Solution II contained cysteic acid, aspartic acid, glutamic acid, serine, threonine, alanine, tyrosine, valine, leucine, histidine and methionine sulphone.

Analytical methods

For total nitrogen the samples were digested for 2 hr with 1 ml conc. H_2SO_4 and 500 mg of a catalyst mixture containing selenium (CHIBNALL, REES and WILLIAMS, 1943). Ammonia was distilled using an all-glass apparatus similar to that described by MARKHAM (1942) and collected in a receiving flask containing 5 ml boric acid solution (4%), 10 ml distilled water and 1 drop (about 0·03 ml) of the mixed indicator (BLOCK and BOLLING, 1951). Phosphorus was measured as described by SPERRY (1942) and α-amino acid nitrogen by the ninhydrin method of VAN SLYKE, DILLON, MACFAYDEN and HAMILTON (1941). Amino acids and ethanolamine were measured by quantitative paper chromatography as described below. Inositol was detected on paper chromatograms by spraying with alkaline silver nitrate reagent (TREVELYAN, PROCTER and HARRISON, 1950).

Animals

Wistar albino rats weighing 175–354 g were used. The animals were decapitated under ether anaesthesia. Brains were rapidly taken out, blotted with Whatman No. 50 paper to remove blood, weighed and then subjected to one of the two following procedures.

Tissue fractionation

All volumes refer to approximately 1 g fresh tissue.

Method 1. *Lipid extract* 1. The tissue was dispersed in 19 ml of chloroform–methanol (2:1) with a Lourdes homogenizer (Lourdes Instrument Corp., New York). The resulting suspension was left standing for 30 min at room temperature, then centrifuged and filtered through Whatman No. 30 paper. The residue was re-extracted twice with 10 ml of the same solvent. The combined lipid extracts were washed as described by FOLCH, LEES and SLOANE STANLEY (1957). The upper phase containing free amino acids and other non-lipid contaminants (GAITONDE, 1961), was discarded and the lower phase was washed twice with 15 ml of the upper phase of the solvent. The washed lower phase contained only traces of glutamic acid and alanine. It was dried at room temperature and the residue was put into a cellophane bag with 10–12 ml of water and 5–6 ml of chloroform–methanol. After dialysis of the emulsion for 24 hr against running tap water, the contents of the dialysis bag were transferred to a boiling tube (25 × 150 mm) and dried in a boiling water bath.

Method 1. *Phosphatido-peptide fraction* 1. The residue remaining after chloroform–methanol extraction was washed 4 times with 15 ml of 10% TCA to remove acid soluble tissue components. The TCA precipitate was then suspended in 15 ml of chloroform–methanol–conc. HCl (200:100:1 by vol) and centrifuged for 1 hr at low speed. The solution was separated from the floating gel by filtration through Whatman No. 1 paper. The gel was extracted twice with 10 ml portions of the same solvent and the combined extracts were dried on a boiling water bath. The residue was designated as 'phosphatido-peptide fraction 1'.

Method 2. *Lipid extract* 2. The fresh tissue was dispersed in 10 ml of 10% TCA with a Lourdes homogenizer. After 15 min at room temperature the suspension was centrifuged and the supernatant solution was discarded. After three further washings under similar conditions, the residue was dehydrated with 20 ml acetone and the acetone extract was collected. The precipitate was then extracted 3 times with 20 ml of chloroform–ethanol (1:1) at room temperature, then once with the same solvent at boiling temperature and finally with 20 ml of ethyl ether. The acetone, chloroform–ethanol and ether filtrates were combined, dried at room temperature, then dialysed and dried as described for Lipid extract 1.

Method 2. *Phosphatido-peptide fraction* 2. The residue remaining after lipid extraction was treated with chloroform–methanol–HCl mixture as described under Method 1. The extract was less viscous and showed no gel formation on centrifugation. It was designated as 'phosphatido-peptide fraction 2'.

Hydrolysis. The dry residues of lipid extracts and phosphatido-peptide fractions were hydrolysed with 10 ml of 6 N-HCl in sealed tubes at 105° for 24 hr. The hydrolysate was filtered and the excess HCl was removed by distillation; the residue was dissolved in water, the solution was made up to 25 ml, and portions were taken for analysis.

Group separation of amino acids on Dowex–1

As basic amino acids travel close to ethanolamine and the separation of histidine from lysine is unsatisfactory on two-dimensional paper chromatograms (Fig. 1), and as ethanolamine and arginine were present in large amounts in the lipid extracts, it was necessary to make a partial separation of the basic compounds from other amino acids in lipid hydrolysates. For this purpose 5–10 ml of the lipid hydrolysate equivalent to 0·5 g fresh tissue was transferred to a Dowex–1 column (15 × 0·9 cm) in the carbonate form. Using moderate air pressure, the effluent was collected together with 40 ml water washings of the resin. These washings contained lysine, arginine, ethanolamine, hexosamine and cations. The neutral amino acids and histidine were eluted from the column with 0·1 N-HCl until the eluate gave a strongly acid reaction to indicator paper. At this stage glutamic and aspartic acids emerge with free hydrochloric acid. The elution of the two amino acids was completed with the same additional amount of 0·1 N-HCl. The two dicarboxylic acids can be collected separately from the neutral amino acids by starting at the first drop of HCl emerging from the column. The approximate volume of acid required to neutralize the known length of the resin column was predetermined to facilitate the operation of the column. The washings, which were acidified, and the eluate were evaporated to dryness. Part of each of the two fractions (0·2 g fresh tissue) was used for paper chromatography.

Quantitative paper chromatography of amino acids

Two-dimensional paper chromatography was carried out on Whatman No. 1 paper (22 in. × 18 in.) in stainless steel cabinets using acetic acid–butanol–water (1:4:5 by vol; upper phase used) as the first solvent and 80% aqueous phenol (in the presence of traces of NH_3) as the second solvent. The papers were dried for 18–24 hr at room temperature in a fume cupboard until practically free from phenol and heated in an oven (mechanical convection type, Reco Model C–425) at 40–50° for 90–120 min. The papers were immediately sprayed with ninhydrin solution under nitrogen pressure, and heated in the same oven at 60° for 20 min. The amino acid spots and two paper blanks of equal area were cut, folded and eluted by gently mixing with 5 ml of 60% ethanol in test tubes. The optical density was measured at 570 mμ within one hour of elution. For quantitative determination of amino acids of lipid hydrolysates the prepared standard solutions of amino acids (25–50 μg each) were subjected to two-dimensional chromatography under the same conditions.

For the determination of methionine and cystine, another portion of the eluate was transferred on to paper and treated with 10 μl of 0·2% ammonium molybdate and 20 μl of 30% H_2O_2 to convert methionine to its sulphone and cystine to cysteic acid. After removing the excess hydrogen peroxide with a jet of hot air, the chromatograms were developed and the two amino acids were determined as described above.

The separation of a mixture of several amino acids on two-dimensional paper chromatograms is shown in Fig. 1. From the data of MEYER (1957) the reaction of α-amino acid with ninhydrin should give an optical density of 0·282 per μg α-amino acid N in 5 ml solution at 575 mμ. The mean optical densities of most of the α-amino acids listed in Table 1 were within 77–96% of this expected value. Duplicate chromatograms of amino acids gave reproducible relative optical densities, but the absolute densities varied to some extent. To obtain good resolution and reproducible optical densities on paper chromatograms prepared by this method the following points should be observed: (1) The separation of amino acids may be increased by allowing the solvent to drip off the lower serrated edge along its length during irrigation with the acetic acid–butanol–water system. (2) The chromatograms were dried in an oven at 40° for 2 hr to remove acetic acid in order to check the formation of 'ghost' spots during the phenol run. The tendency to form 'ghost' spots was greater if phenol containing a preservative H_2PO_3 was used. There were advantages in using 85% phenol instead of 80% phenol provided the temperature of the chromatographic cabinet could be maintained at 23–26°. Under these

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conditions the separation of glycine from serine was complete and the time required for irrigation was considerably shortened. (3) For optimal reaction of ninhydrin with amino acids on paper chromatograms it was important that the chromatograms were dried until free from phenol. High paper blanks and low optical densities of amino acids were invariably due to traces of phenol on paper chromatograms. (4) The rate of reaction of ninhydrin with amino acids on paper chromatograms was more rapid when the spray was prepared with a citrate buffer of pH 7·5 than when prepared with a citrate buffer of pH 5·0. The optical densities, although reproducible at either value, were higher at pH 7·5. (5) Under

FIG. 1.—Two-dimensional paper chromatogram of amino acids.

1. Aspartic acid
2. Thiolhistidine
3. Glutamic acid
4. Serine
5. Glycine
6. Threonine
7. α-Alanine
7a. β-Alanine (blue)
8. Tyrosine
9. Valine, Tryptophan, Methionine
10. Phenylalanine
11. Leucine and *iso*leucine
12. Cystine
12a. Cysteic acid
13. and 13a. DL-*allo*-cystathionine
13a. L-*allo*-cystathionine
14a. Homocysteic acid
17. Lysine
18. Histidine
19. Arginine
20. Methionine sulphone
20a. Methionine sulphoxide
21. Hydroxyproline (pink)
22. Ethanolamine
23. Methyl histidine (bluish-purple)
24. Histamine
25a. Ethionine sulphoxide
26. γ-Aminobutyric acid
26a. α-Aminobutyric acid
27. Proline (yellow)
28. Glutathione
29. N-Acetyl aspartic acid
30. Taurine
31. Dihydroxyphenylalanine
32. Homoserine
33. Asparagine
34. Glutamine / Hexosamine (glucosamine) / Citrulline

optimal conditions the optical density of paper blanks (18–20 sq. cm) taken from the right hand area (Fig. 1) of different batches of chromatograms varied from 0·03 to 0·05; higher blanks (0·06–0·08) were obtained for the left hand area in the region of the origin. Hence for amino acids in these two regions the corresponding paper blanks were used to obtain the net optical density of the amino acid spot.

Paper chromatography of inositol. Parts of the hydrolysate of the phosphatido-peptide fractions (0·2 g fresh tissue) and of the residual tissue proteins (10 mg) were deionized using a column of mono-bed resin (Amberlite, MB–1). The aqueous washings containing inositol and other neutral compounds

TABLE 1.—OPTICAL DENSITIES OF AMINO ACIDS AFTER TWO-DIMENSIONAL PAPER CHROMATOGRAPHY

Substance	Optical density/μg amino N mean $\pm$ S.D.	Per cent
Aspartic acid	0·173 $\pm$ 0·008	61
Glutamic acid	0·266 $\pm$ 0·012	94
Serine	0·226 $\pm$ 0·003	80
Glycine	0·089 $\pm$ 0·009	32
Threonine	0·216 $\pm$ 0·002	77
Alanine	0·273 $\pm$ 0·010	97
Tyrosine	0·160 $\pm$ 0·005	57
Valine	0·275 $\pm$ 0·011	98
Leucine	0·271 $\pm$ 0·008	96
Phenyalanine	0·170 $\pm$ 0·009	60
Histidine	0·151 $\pm$ 0·002	54
Lysine	0·243 $\pm$ 0·007	86
Arginine	0·219 $\pm$ 0·007	78
γ-Aminobutyric acid	0·216 $\pm$ 0·009	77
Methionine sulphone	0·234 $\pm$ 0·006	83
Cysteic acid	0·164 $\pm$ 0·020	58
Taurine	0·137 $\pm$ 0·009	49
Ethanolamine	0·087 $\pm$ 0·006	31

Paper chromatograms were sprayed with ninhydrin solution and the spots eluted in 5 ml of 60% ethanol. The optical densities were measured in a 1 cm thick Beckman cell at 570 mμ. The figures in the last column are percentages of 0·282, the theoretical optical density per μg amino acid nitrogen in 5 ml solution at 575 mμ calculated from the data of MEYER (1957).

were collected, evaporated to dryness and transferred on to Whatman No. 1 paper. The chromatograms were developed with a mixture of acetone and water (90:10) and sprayed with silver nitrate reagent (TREVELYAN *et al.*, 1950).

RESULTS

Composition of lipid extracts prepared by two different procedures

Tables 2 and 3 show the total phosphorus, nitrogen, α-amino nitrogen, ethanolamine and amino acids of hydrolysates of lipid extracts prepared from rat brain by (a) chloroform–methanol extraction (Method 1) and (b) extraction of a TCA precipitate with acetone, chloroform–ethanol and ether in succession (Method 2). There was little difference in the phosphorus and ethanolamine content of the two lipid extracts: the total amount of lipid extracted by each method was therefore, similar. The total nitrogen and α-amino nitrogen contents were higher in extracts prepared by Method 2: the data suggest that about 15 per cent more protein was extracted from the TCA precipitate by organic solvents than was obtained by extracting the fresh tissue with chloroform–methanol.

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TABLE 2.—COMPOSITION OF LIPID EXTRACTS OBTAINED BY TWO DIFFERENT PROCEDURES

Rat wt (in g) and sex	Method	Total nitrogen	α-Amino nitrogen	Total phosphorus	Ethanolamine nitrogen
		(μg per g fresh brain)			
213 (F)	1	1492	518	1860	202
317 (M)		1403	411	1885	245
210 (F)	2	1575	648	1835	232
354 (M)		1534	468	1795	195

In Method 1 lipid extracts were prepared by treating the fresh tissue with chloroform–methanol and in Method 2 lipid extracts were obtained from the TCA precipitate of the fresh tissue by successive treatment with acetone, chloroform–ethanol and ether.

TABLE 3.—AMINO ACID COMPOSITION OF PROTEOLIPID FRACTIONS OF RAT BRAIN OBTAINED BY TWO DIFFERENT METHODS

	(μg α-amino N/g fresh tissue)	
	Method 1	Method 2
Total nitrogen	1492	1575
Total α-NH_2 nitrogen	518*	648*
Aspartic acid	17·2 (3·3)	36·5 (5·6)
Glutamic acid	21·4 (4·1)	45·7 (7·1)
Serine	91·8 (17·7)	99·8 (15·4)
Glycine	52·5 (10·1)	52·9 (8·2)
Threonine	31·2 (6·0)	26·1 (4·0)
Alanine	46·2 (8·9)	44·6 (6·9)
Tyrosine	14·5 (2·8)	13·4 (2·1)
Valine	28·0 (5·4)	32·9 (5·1)
Phenylalanine	26·3 (5·1)	18·0 (2·8)
Leucine + *iso*leucine	74·3 (14·3)	75·5 (11·7)
Arginine	20·1 (3·9)	33·8 (5·2)
Lysine	12·9 (2·5)	27·0 (4·2)
Histidine	12·9 (2·5)	3·6 (0·6)
Methionine	1·7 (0·3)	3·9 (0·6)
Cystine	4·0 (0·8)	2·9 (0·4)
Proline	+	+
Total α-NH_2 nitrogen recovered	455·0	516·6

* Determined by the ninhydrin method of VAN SLYKE *et al.* (1941).

Figures in parentheses represent the quantity of the α-amino acid nitrogen as percentage of the total found by the ninhydrin method of VAN SLYKE *et al.* (1941).

Two-dimensional paper chromatography revealed differences in the amino acid composition of proteins present in the two lipid extracts (Table 3). About 80–87 per cent of the α-amino acid nitrogen present in the original samples was accounted for by the amino acids listed in Table 3; tryptophan, which was destroyed during

hydrolysis, proline and amide nitrogen were not determined. The amino acid composition when expressed as percentage of the total α-amino acid nitrogen/g fresh tissue in the two lipid hydrolysates showed that there was more glutamic acid (73%), aspartic acid (70%), arginine (33%), lysine (68%) and methionine (100%) in lipids extracted by Method 2. The percentage of most of the other amino acids was, however, less in lipids extracted by Method 2.

Composition of 'phosphatido-peptide fractions' obtained by two different methods

The total phosphorus content of the two phosphatido-peptide fractions was of the same magnitude irrespective of the previous treatment of the tissue residue (Table 4).

TABLE 4.—COMPOSITION OF PHOSPHATIDO-PEPTIDE FRACTIONS PREPARED BY TWO DIFFERENT PROCEDURES

Rat wt (in g) and sex	Method	Total nitrogen	Total phosphorus	Ethanolamine nitrogen
		(μg per g fresh brain)		
175 (F)	1	735	165	10
317 (M)		777	169	13
210 (F)	2	257	154	11
354 (M)		282	186	13

Phosphatido-peptide fractions were obtained by treatment of the tissue residues remaining after lipid extraction as described in the footnote of Table 2.

The fractions obtained by both methods also contained bound inositol. Since the tissue residue remaining after extraction with acidified chloroform–methanol also contained bound inositol it may be inferred that the extraction of phosphatido-peptide was not complete under the conditions of either of the methods used.

Considerable differences were observed in the total nitrogen content of the two phosphatido-peptide fractions: Fraction 1 contained approximately 3 times as much nitrogen as Fraction 2. Ethanolamine accounted for 1·3–1·6 per cent of the total nitrogen of the peptide obtained by Method 1, and for 4·3–4·6 per cent of the total nitrogen of the corresponding fraction obtained by Method 2.

Amino acid composition

The difference in the total nitrogen content of the two phosphatido-peptide fractions was due mainly to the greater quantity of peptides extracted by Method 1 (Table 5). About 62 per cent of the total nitrogen of Fraction 1 and 37 per cent of the total nitrogen of the peptide Fraction 2 were accounted for by the α-amino acid nitrogen measured by two dimensional paper chromatography. Except for cystine and methionine the amounts of amino acids in peptide Fraction 1 were 4 to 6 times higher than in peptide Fraction 2. However, the amino acid composition, when expressed as percentage of the total α-amino acid nitrogen recovered by paper chromatography, gave a similar pattern of distribution for most amino acids except glutamic acid, serine, phenylalanine-leucine, and cystine (Table 5).

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Paper chromatography of the hydrolysates of the two peptide fractions showed the presence of a blue spot above the α-alanine spot after spraying with the ninhydrin reagent. Several other amino acids were therefore chromatographed and it was found that β-alanine, which also gave a blue spot, had the same R_F as that of the unknown compound in the peptide fraction.

TABLE 5.—AMINO ACID COMPOSITION OF PHOSPHATIDO-PEPTIDE FRACTIONS OF RAT BRAIN OBTAINED UNDER DIFFERENT CONDITIONS

	(μg α-amino N/g fresh tissue)	
	Method 1	Method 2
Total nitrogen	735	257
Aspartic acid	46·5 (10·3)	8·5 (9·0)
Glutamic acid	51·2 (11·3)	7·2 (7·6)
Serine	23·6 (5·2)	10·0 (10·5)
Glycine	28·6 (6·3)	6·8 (7·2)
Threonine	21·9 (4·9)	4·2 (4·4)
Alanine	35·0 (7·8)	7·5 (7·9)
Tyrosine	11·6 (2·6)	2·1 (2·2)
Valine	30·9 (6·9)	5·7 (6·0)
Phenylalanine, Leucine	121·0 (26·8)	19·5 (20·6)
Arginine, Lysine, Histidine	58·1 (12·9)	12·2 (12·9)
Methionine	10·9 (2·4)	1·3 (1·4)
Cystine	13·0 (2·9)	9·7 (10·2)
Proline	+	+
Total α-NH_2 nitrogen recovered	452·3	94·7

Figures in parentheses represent the quantity of α-amino acid nitrogen as percentage of the total α-amino acid nitrogen determined by paper chromatography.

DISCUSSION

Evidence was obtained that the amounts of protein extracted by organic solvents from the TCA precipitate of rat brain (Method 2) are slightly greater than the amounts obtained by treatment of the fresh tissue with chloroform–methanol (Method 1). The data on total phosphorus and ethanolamine, however, suggested that the two methods were equally efficient in lipid extraction. The greater amount of α-amino acid nitrogen in the fraction prepared by Method 2 was due mainly to the presence of larger amounts of bound aspartic acid, glutamic acid, methionine, arginine and lysine. The preferential extraction of proteins rich in these amino acids by Method 2 indicated that different proteins were extracted under the conditions of the two methods. Although by definition proteolipids are obtained from fresh tissues by treatment with chloroform–methanol, it is likely that the proteins of lipid extracts prepared by Method 2 also contain (a) proteolipids, (b) other lipoproteins and (c) proteins soluble in organic solvents in the presence of acids such as trichloroacetic acid (MICHAEL, 1958). It has been suggested that acids dissociate salt-like compounds

normally formed by phosphatido-peptides with the tissue proteins so that the phosphatido-peptides are rendered soluble in chloroform–methanol–HCl (FOLCH, 1952). If this is so, it is possible that phosphatido-peptides may be extracted to some extent by acetone from the TCA precipitate. The two phosphatido-peptide fractions described in this work contained phosphorus, nitrogen, inositol and peptides, and in this respect they resemble the phosphatido-peptide fraction described by FOLCH and LEBARON (1953). It is difficult to compare these two phosphatido-peptide fractions with that described by FOLCH and LEBARON since in the present investigation the tissue residues were pre-treated with TCA: but FOLCH and LEBARON used the trypsin-resistant tissue residue to obtain phosphatido-peptides. The peptide nitrogen content of Fraction 1 was 3 times that of Fraction 2. The presence of bound inositol in the proteins remaining after treatment of the TCA precipitate with acidified chloroform–methanol clearly showed that phosphatido-peptides were not extracted quantitatively in either of the fractions described in this work. The presence of methionine and β-alanine in the two fractions suggest that phosphatido-peptide Fractions 1 and 2 probably contained other peptides or soluble proteins besides the phosphatido-peptides described by FOLCH and LEBARON. It may be concluded that organic solvents extract different proteins from the tissue under different conditions.

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SUMMARY

The total nitrogen, α-amino acid nitrogen, phosphorus and amino acids were determined in brain lipoprotein fractions prepared (a) by homogenizing the tissue with a mixture of chloroform–methanol (Method 1) and (b) by extracting the TCA precipitate of brain tissue successively with acetone, chloroform–ethanol and ether (Method 2). The proteins extracted by Method 2 differed qualitatively from proteolipids obtained by Method 1: the proteolipids contained more glutamic acid (73%), aspartic acid (70%), arginine (33%), lysine (68%) and methionine (100%).

Phosphatido-peptide fractions obtained by extracting tissue residues under different conditions with chloroform–methanol–HCl were also examined.

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THE SUBCELLULAR LOCALIZATION OF GLUTAMIC DECARBOXYLASE IN RAT BRAIN

SØREN LØVTRUP
Department of Neurobiology, University of Göteborg, Sweden

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THE DEMONSTRATION of glutamic decarboxylase in brain (ROBERTS and FRANKEL, 1951) led to the discovery of the following metabolic pathway in brain tissue: Glutamate → γ-aminobutyrate → succinic semialdehyde → succinate (BESSMAN, ROSSEN, and LAYNE, 1953; ROBERTS and BREGOFF, 1953). According to MCKHANN and TOWER (1961) this reaction sequence takes place in cat brain cortex mitochondria. Oxidation of γ-aminobutyrate by rat brain mitochondria was reported by SACKTOR, PACKER, CUMMINS, and HACKLEY (1960), but preliminary experiments in this laboratory with rat brain mitochondria did not support this finding. The present experiments were therefore made to ascertain whether or not glutamic acid may be metabolized through decarboxylation by rat brain mitochondria.

EXPERIMENTAL

Fractionation procedure. A 15 per cent rat brain homogenate in 0·44 M-sucrose was prepared (6 brains in approximately 50 g sucrose solution). A portion was taken for enzyme determination, and in the first stage the remaining homogenate was centrifuged in a Spinco centrifuge (rotor SW 25·1) at 5000 rev/min (average 2100 **g**) for 10 min. The solid residue was resuspended in sucrose and centrifuged again to obtain the nuclear fraction; to avoid too high dilution in the final supernatant, the supernatant fluid from the nuclei was discarded. The supernatant fluid from the first stage was centrifuged at 1300 rev/min (average 14500 **g**) for 15 min. The precipitate was washed three times with sucrose, and each time was centrifuged at 9000 rev/min (average 7000 **g**) for 15 min. The precipitate from the last centrifugation was then suspended in sucrose to a total weight of 6 g to give the mitrochondrial fraction. The supernatant fluids from the first centrifugation and first washing of the mitochondrial fraction were combined, and centrifuged at 25000 rev/min (average 53500 **g**) for 1 hr. The precipitate, resuspended in sucrose to a total weight of 8 g, was the microsomal fraction. The final supernatant fluid was weighed and used directly as a fourth subcellular fraction. The mitochondrial fraction was found to be of satisfactory purity by electron microscopy (in collaboration with Dr. TOR ZELANDER).

Manometric technique. The medium in the Warburg vessels contained 100 μl of 0·133 M-$MgSO_4$, 100 μl of 0·133 M-K phosphate buffer (pH 7·4), 100 μl of 0·56 M-glucose, 100 μl of 0·02 M-ATP, 0·75 mg hexokinase (Worthington, fraction IV), 100 μl of 0.26 M-substrate, 500 μl of the mitochondrial suspension, containing mitochondria from 500 mg of brain, and sucrose to 2 ml. The measurements were carried out at 37°. These experimental conditions are optimal for brain mitochondria. P/O values close to 3 are obtained with pyruvate or glutamate.

Enzyme determination. The procedure described by ROBERTS and FRANKEL (1951) was employed with the only modification that 500 μg pyridoxal phosphate was added to each vessel. One ml of the various fractions was used in each assay. The determinations were carried out at 30°.

RESULTS

Preliminary experiments (Table 1) indicated that γ-aminobutyrate is not oxidized by the mitochondria. Malate was included as it was used for fortification of the media in the experiments described by TOWER (1959). The results with glutamate and pyruvate are included to show that the mitochondria were active.

Since the decarboxylase is present in brain tissue, it was decided to determine the subcellular localization of the enzyme.

The results obtained in two experiments (Table 2) show that a fairly considerable amount of enzyme was found in the nuclear fraction. It can hardly be doubted that most of this enzyme activity represents the activity of unbroken cells. The enzyme content of the mitochondrial fraction was quite low. The significance of this finding

TABLE 1.—OXIDATION OF SUBSTRATES BY RAT BRAIN MITOCHONDRIAL PREPARATION

	Glutamate	Pyruvate + malate (1 μM)	γ-Amino-butyrate	γ-Amino-butyrate + malate (2 μM)	Malate (2 μM)
μatoms oxygen per hr per mg N	14·1	41·6	0	5·0	8·0

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TABLE 2.—ENZYMIC ACTIVITY OF SUBCELLULAR FRACTIONS

	Glutamic decarboxylase activity			
	Experiment 1		Experiment 2	
	μmoles CO_2 per hour	Activity per cent	μmoles CO_2 per hr	Activity per cent
Whole homogenate	118·0	(100)	122·0	(100)
Nuclear fraction	24·4	20·7	29·2	19·8
Mitochondrial fraction	4·0	3·4	5·2	4·3
Microsomal fraction	26·4	22·4	31·4	25·8
Supernatant	41·8	34·6	40·5	33·2
Sum of fractions		81·1		83·1

will be dealt with in the discussion. Both the microsomal fraction and final supernatant fluid contained quite large amounts of enzyme. The microsomal fraction was not washed, and it is therefore reasonable to assume that some of the activity of this fraction rightly belongs to the supernatant fraction. The activity in the mitochondrial fraction of Experiment 2 corresponds to 2·4 μmoles CO_2 per hr per mg N. This rate is low compared with the rate at which glutamate is oxidized by our mitochondrial preparations (Table 1), but it should be recalled that the experimental temperature differed in the two sets of experiments.

DISCUSSION

Only a very small fraction of the total glutamic decarboxylase of rat brain can be recovered in the mitochondria. The enzyme content of the whole homogenate could not be accounted for quantitatively in the fractions, and the possibility exists

that most of the lost activity was located in the mitochondria, which were more carefully washed than the other fractions. However, the mitochondria are of course not completely pure, and it may just as well be that the small enzyme content represents impurities. Irrespective of whether the mitochondria proper contain a substantial amount of enzyme, or none at all, the fact remains that glutamic acid is oxidized by the mitochondria at a much higher rate than that at which it is decarboxylated. Taken together with the observation that γ-aminobutyrate is not oxidized by rat brain mitochondria, this result therefore seems to exclude that glutamic acid is oxidized via γ-aminobutyrate to succinate in rat brain mitochondria. The disagreement between the present results, and those obtained by McKHANN and TOWER may be due to the admitted presence of microsomes in their mitochondrial preparations.

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ACTIVATION AND INHIBITION OF ADENOSINE TRIPHOSPHATASES OF SUBCELLULAR PARTICLES FROM THE BRAIN

D. H. DEUL* and H. MCILWAIN
Department of Biochemistry, Institute of Psychiatry,
(British Postgraduate Medical Federation, University of London)
Maudsley Hospital, S.E.5

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EXPECTATION that the adenosine triphosphatases of neural tissues, like those of muscle, might participate in the tissue's utilization of metabolically derived energy (see for example GORE, 1951; MCILWAIN, 1957) were supported by the findings of SKOU (1957, 1960) and of HESS and POPE (1957), that the adenosine triphosphatases of crab nerve and rat brain required the presence of sodium salts for maximal activity. The enzymes also showed the requirements for magnesium and potassium salts which are more usual in reactions involving adenosine triphosphate (LOWENSTEIN, 1958, 1960); the sodium requirement might connect the enzymes with the performance of osmotic work in neural tissues.

Recent studies from these laboratories have shown that ion movements in cerebral tissues can be modified by substances which include basic proteins, gangliosides and suramin (WOODMAN and MCILWAIN, 1961; MCILWAIN, WOODMAN and CUMMINS, 1961; and unpublished). The effects of these agents on the adenosine triphosphatases of cerebral tissues have therefore been examined. The microsomal fraction of ground cerebral tissues contains much of the tissues' gangliosides and is involved in the interactions of basic proteins and gangliosides with the tissue (WOLFE and MCILWAIN, 1961; WOLFE, 1961). JÄRNEFELT (1960) showed that microsomal fractions of ground cerebral tissues contained an adenosine triphosphatase activated by sodium salts, but the degree of activation was not large. During the present studies attention has therefore been paid to the conditions under which the sodium activation is manifested, and also to the enzyme level and to the degree of sodium activation in different subcellular fractions of the ground tissue.

EXPERIMENTAL

Cerebral preparations. Adult guinea pigs of either sex were struck on the back of the neck, exsanguinated, and the cerebrum placed on filter paper moistened with 0·32 M-sucrose in an ice-cold Petri dish within 2 min of striking the animal. The cerebrum was then opened immediately and all except the grey matter of the cortex removed. The cortex was weighed and usually found to be about 2 g and was ground in 25 ml of 0·32 M-sucrose at 0° in a test-tube homogenizer with a plastic plunger. The suspension was transferred to the chamber of a piston-press homogenizer (EMANUEL and CHAIKOFF, 1957, 1960; for use with cerebral tissue see WOLFE and MCILWAIN, 1961), also at 0–4° and fitted with a plunger of 25 μ clearance. The press was immediately operated and the suspension collected in a cooled container. Of the suspension, still at 0°, 2·5 ml were taken as a sample and the remainder

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placed in two tubes of a No. 40 head of a Spinco preparative ultracentrifuge which was taken to 800 **g** for 10 min. The precipitates were made up to 10 ml with 0·32 M-sucrose and centrifuged as before. The resulting precipitate was made up to a total volume of 10 ml in 0·32 M-sucrose, and stored in this form. The supernatant liquid and washings were taken to 5000 **g** for 10 min and the precipitates combined and again washed wit h sucrose, centrifuging at the speed of preparation, and again resuspended and stored. These operations were repeated at 20,000 **g** with the supernatants and were followed by centrifuging at 104,500 **g** for 95 min; this precipitate was suspended in 10 ml sucrose without washing. Microscopic examinations of the fractions gave results similar to those of WOLFE (1961) and are summarized in Table 1. Fractions were stored at 0–4° as sucrose suspensions.

Adenosine triphosphatase assay. Duplicate reaction mixtures of 2 ml were prepared in 10 ml tubes and contained, except when otherwise specified 25 mM-2-amino-2-hydroxymethylpropane-1 : 3-diol (tris) HCl at pH 7·4; enzyme preparation contributing 0·05–0·2 mg protein/ml; 3 mM-adenosine triphosphate (ATP) as its tris salt (see below); and the activators or inhibitors under investigation. The tubes, lacking only ATP, were placed at 37° for 5 min, the ATP was added, and 15 min later the tubes were placed in ice–water, and 1 ml of 40% (w/v) trichloroacetic acid was added. After 20 min at 0° the tubes were centrifuged at 850–1000 **g** for 2–3 min and two samples of 1 ml were taken for determination of inorganic phosphate. For inorganic phosphate the method of BERENBLUM and CHAIN as modified by MARTIN and DOTY and stated by LINDBERG and ERNSTER (1956) was used with minor adaptations: to the mixture of H_2SO_4, ammonium molybdate, silicotungstic acid and isobutanol-benzene in a glass stoppered tube was added the 1 ml sample. The tube with others was shaken for 15 sec and centrifuged. To 2 ml of its upper layer 3 ml of the H_2SO_4–ethanol was added and colour intensities were read at 650 mμ.

Other determinations. The protein content of the sucrose suspensions was determined in 0·1 ml samples according to LOWRY, ROSEBROUGH, FARR and RANDALL (1951). The standard curve was constructed using crystalline bovine plasma albumin in the presence of sucrose in a concentration equal to that contributed by the tissue suspensions, as sucrose depressed the readings.

Reagents. Tris ATP with little Na was prepared from the disodium salt (Sigma) with Dowex-50 resin in the H^+ form (JÄRNEFELT, 1961). The Na-ATP (455 mg) in water (20 ml) was stirred at 0° with the resin (about 2·5 g) for 15 min and filtered, the filter was washed with a few ml of water and the filtrate with washings was immediately taken with 0·5 M-tris base to pH 7·4. Solutions so prepared were diluted to 15 mM in ATP and were found to be 0·85 mM in Na, and thus contributed 0·17 mM-Na to reaction mixtures. Ganglioside preparations were made and their *N*-acetylneuraminic acid determined as described by MCILWAIN (1961*a*), giving the following values: Sample A, 26%; sample B (re-dialysed), 28%; sample C 28%. We are indebted to Mr. D. A. BOOTH and Dr. D. B. GAMMACK for these samples and determinations. Suramin, the Na salt of bis-(*m*-aminobenzoyl-*m*-amino-*p*-methylbenzoyl-1-naphthylamine-4:6:8-trisulphonate) carbamide, was from Imperial Chemical Industries (Pharmaceuticals) Ltd. Protamine was the analysed specimen of clupein sulphate described by MCILWAIN (1961*a*).

RESULTS

Phosphate liberation by five fractions

Table 1 shows the fractions employed; the protein of the tissue was fairly evenly divided among them. In studying their ability to liberate inorganic phosphate from adenosine triphosphate, initial conditions were chosen from those of the investigations cited above, and either Ca or Mg was employed as the divalent ion in a concentration equimolar with the adenosine triphosphate. The further addition of NaCl plus KCl was then found to alter relatively little the phosphate liberated in the presence of Ca (change, −35 to +45%) but to stimulate markedly that liberated in the presence of Mg. Greatest stimulation was found in the microsomal fraction, of which the activity was increased nearly 3-fold by the Na and K. The greatest total activity was found in the mitochondrial fraction (3), the microsomal fraction (5), and in an intermediate fraction (4) which contained both types of particles. The mitochondrial (3) and microsomal (5) fractions have been made the subject of the following more detailed

TABLE 1.—PREPARATION, CONSTITUENTS, AND ADENOSINE TRIPHOSPHATASE ACTIVITY OF FRACTIONS FROM GUINEA PIG CEREBRAL CORTEX

Fraction separated at g-min	Components	Protein (mg/ fraction)	Adenosine triphosphatase activity (μmoles PO_4/hr) with:									
			(*a*) Ca		(*b*) Ca, Na, K		$\frac{(b)}{(a)} \times 100$	(*c*) Mg		(*d*) Mg, Na, K		$\frac{(d)}{(c)} \times 100$
			per mg protein	per fraction	per mg protein	per fraction		per mg protein	per fraction	per mg protein	per fraction	
1. (Whole homogenate)	—	186	10·8	2010	11·7	2180	108	12·6	2340	20·8	3870	165
2. 8,000	Nuclei (blood vessel and myelin fragments)	31	7·9	245	10·1	313	127	8·1	251	18·4	572	228
3. 50,000	Mitochondria (small glial nuclei and myelin)	37	9·1	337	5·8	214	65	14·6	540	18·0	667	123
4. 200,000	Smaller mitochondria and larger microsomes	41	10·8	443	11·5	472	107	11·3	463	23·0	943	204
5. 10,000,000	Microsomal and post-microsomal particles	27	19·1	517	27·5	743	145	14·4	389	42·5	1148	295
6. Supernatant	—	29	8·8	254	6·0	173	68	9·6	276	8·2	246	85
7. (Sum of fractions)		165	—	1796	—	1915	—	—	1919	—	3576	—
8. Recovery of fractions as % of whole homogenate		89	—	89	—	88	—	—	82	—	92	—

Fractions were examined with a phase-contrast microscope; lesser components are included in parentheses.
Adenosine triphosphatase activities were determined after preparations had been kept at 0° for 7 days.
Reagents used (mM): tris-ATP, 3; $CaCl_2$,3; $MgCl_2$, 3; KCl, 30; NaCl, 200; tris, 125; the metal salts being added as indicated in the column headings.

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studies; it will be noted that they are prepared at very different centrifugal fields and that the intermediate fraction aids in ensuring their distinctiveness.

Adenosine triphosphatase in microsomal and mitochondrial fractions

Effects of H, Mg, *and* Na *ions.* The adenosine triphosphatase activities of the two fractions were found to differ in a number of characteristics. Thus their phosphate

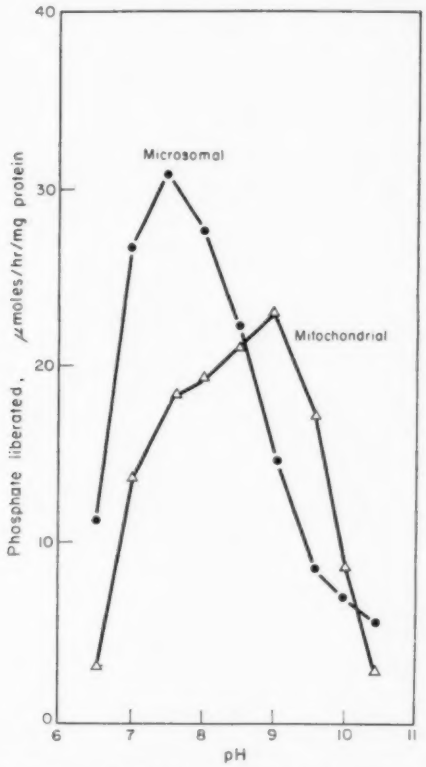

FIG. 1.—The pH dependence of the adenosine triphosphatases of the mitochondrial and microsomal fractions, 1 day after preparation from guinea pig cerebral cortex. Tris was taken to the chosen pH values with HCl and incorporated at 125 mM in reaction mixtures with 3 mM-$MgCl_2$, 20 mM-KCl, 100 mM-NaCl and 3 mM-tris ATP.

liberation from adenosine triphosphate was maximal at different pH values; liberation by the microsomal fraction was optimal at pH 7·5, and the mitochondrial fraction at pH 9 (Fig. 1). Further study of the enzymes was carried out at pH 7·5; this value was chosen because it is closer to that normal to the tissue, and also because the microsomal enzyme was more important to the present study than the mitochondrial.

In solutions containing only tris-ATP and tris-Cl, phosphate liberation by both microsomal and mitochondrial preparations was slow but was greatly accelerated by Mg salts (Fig. 2) . With the microsomal fraction, maximal activation was reached at 2-3 mM-Mg and further Mg did not greatly alter the phosphate liberated. A large and variable acceleration of mitochondrial adenosine triphosphatase activity was effected by Mg salts; it was greater with 1 or 2 mM-$MgCl_2$ than with higher concentrations,

which were inhibitory. In the presence of 3 mM-$MgCl_2$, addition of Na^+ as the only univalent inorganic ion accelerated the activity of both preparations, but to extents which depended on their handling; this is discussed below. With the mitochondrial fraction, activation ranged from the 16 per cent shown in Fig. 2 to about 50 per cent. Activation of the microsomal fraction was always greater than that of the

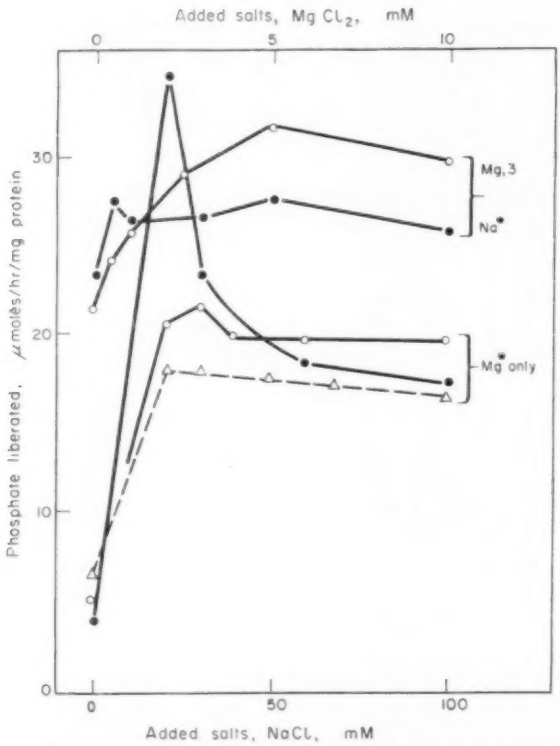

FIG. 2.—Effects of Mg and Na salts on mitochondrial (●) and microsomal (○) adenosine triphosphatases; the ion asterisked was added to give the concentrations shown in the horizontal scales. Preparations had been kept 1 day at 0–4°, except that shown by a dotted line, which was microsomal and had been kept 3 days.

mitochondrial fraction, made and examined at the same time; in the experiments of Fig. 2, it was greater by a factor of 3.

Effects of Mg, Na *and* K *ions.* Comparison of the batches of cerebral preparations which had given the variable results just quoted, suggested their differences to be correlated in part with the length of time that the tissue fractions had been kept before the assays were made. Examination of the combined effects of Mg, Na and K ions within 1 or 2 days of preparation showed (Fig. 3) that, with microsomes examined in the presence of Mg and K, increase in Na led to a moderate (30%) stimulation of activity. A similar increase was shown over the wide range of potassium concentrations from 30 to 240 mM, which were employed. The mitochondrial fraction with 30 mM-K salts changed in activity with increase in Na in the same fashion as did the microsomal preparation; but with KCl, NaCl had no effect or was inhibitory. The higher salt

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concentrations employed are markedly hypertonic but nevertheless showed effects specific to Na or K.

The behaviour of other preparations is shown in Fig. 4. In general, these were preparations which had been kept for a few days at 0°; the age of the preparations has accordingly been noted in reporting data, though this does not appear to be the only factor involved and investigation of the difference is in progress. Na salts are seen (Fig. 4) to activate greatly the release of inorganic phosphate from adenosine triphosphate by microsomal fractions already in the presence of Mg and K, increasing

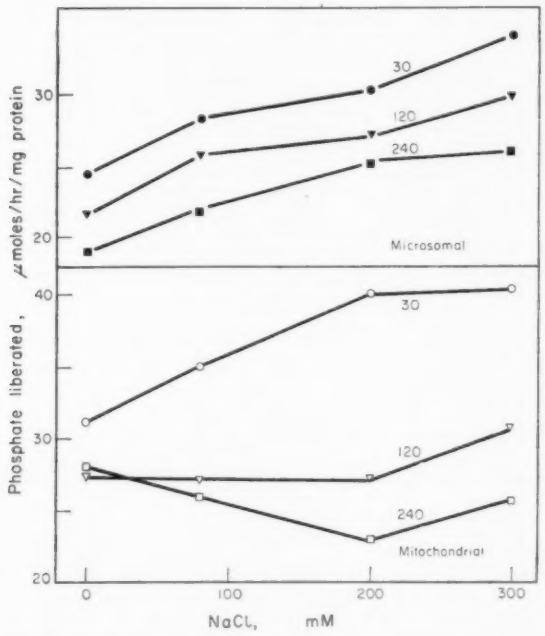

FIG. 3.—Effect of NaCl on the adenosine triphosphatase activities of microsomal and mitochondrial fractions after 2 days at 0–4°, examined with 3 mM-ATP, 3 mM-$MgCl_2$, and the concentrations of KCl shown (mM).

the rate to as much as 270 per cent of its value in the absence of Na. In the same period the activity of the mitochondrial fraction fell by about a third, and was increased only a little by Na. This difference between the two types of particle was repeatedly demonstrated. The Na activation was evident in microsomal fractions kept at 0° for up to 10–14 days; during this time activity declined a little.

Other ions. The tris buffer which was part of most reaction mixtures could be replaced by glycylglycine with only small change in the adenosine triphosphatase activity of the microsomes (Table 2); this replacement necessarily involved change in the anions of the medium, in which chloride normally preponderated. Chloride could however be completely replaced by acetate without change in activity; replacement of most of the chloride by the monomethyl sulphate ion, was also without influence on the phosphate liberation. These latter replacements were made also in the mitochondrial fraction with similar lack of effect (Table 2).

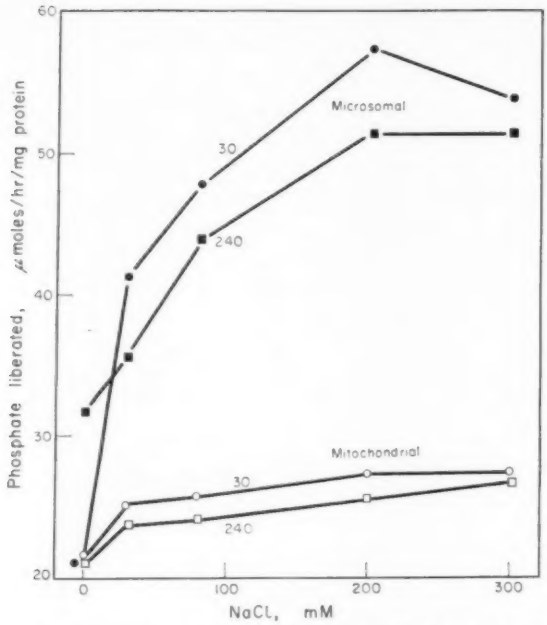

FIG. 4.—As Fig. 3 with preparations kept at 0–4° for 8 days.

TABLE 2.—BUFFERS, ANIONS, AND THE MICROSOMAL AND MITOCHONDRIAL ADENOSINE TRIPHOSPHATASES.

Expt.	Constituents of reaction mixture (mM)					Inorganic phosphate liberated (μmoles/mg protein/hr)	
	Buffer	K	Na	Cl	Other anions	Microsomal fraction	Mitochondrial fraction
A	Tris, 125	100	100	308	0	30·8	20·7
	Tris, 125	100	100	208	Acetate, 100	32·4	20
	Tris, 125	100	100	108	Acetate, 200	33·7	20
	Tris, 125	100	100	0	Acetate, 300	31·9	21·2
	Tris, 125	100	100	208	Methyl sulphate, 100	28·0	20·3
	Tris, 125	100	100	108	Methyl sulphate, 200	28·8	21·4
B	Glycylglycine	30	30	66	0	42·3	—
	Tris, 125	30	30	176	0	39·1	—

All reaction mixtures contained the buffer, pH 7·4, Mg salts at 3 mM and adenosine triphosphate, added last, at 3 mM. Particulate preparations were used in Expts. A, 1 day after their separation and in B, 4 days after separation. Values are the mean of closely-agreeing duplicates.

In the presence of 3 mM-$MgCl_2$, 30 mM-KCl and 33 mM-NaCl in tris buffer, 3–12 mM-$CaCl_2$ was found inhibitory to the adenosine triphosphatase of both microsomes and mitochondria, activity being diminished by up to 30 and 60 per cent respectively. Lithium chloride at 30, 80, 200 and 300 mM was examined for action on the microsomal enzyme in the presence of 3 mM-$MgCl_2$ and 30 mM-KCl. It caused less

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than 10 per cent change in activity in experiments in which 200 mM-NaCl increased activity to 202 and 230 per cent of its original values.

Gangliosides and suramin

These are acidic substances of relatively large molecular weight, and both inhibited the microsomal adenosine triphosphatase (Table 3). Suramin inhibited also the mitochondrial enzyme, which was less affected by ganglioside preparations. On

TABLE 3.—EFFECTS OF SURAMIN, GANGLIOSIDES AND PROTAMINE ON THE ADENOSINE TRIPHOSPHATASE ACTIVITY OF MICROSOMAL AND MITOCHONDRIAL FRACTIONS

Expt.	Additional ions present	Further substances (mg/ml)	Inorganic phosphate liberated (μmoles/mg protein/hr)	
			Microsomal fraction	Mitochondrial fraction
A	None	None	27·3	31
	K, Na	None	39·6	33·8
	K, Na	Suramin, 0·1	29·5	22·8
		Suramin, 0·5	20·6	18·1
		Suramin, 2·5	18·3	18·1
	K, Na	Protamine sulphate, 0·1	45·6	29·6
		" 0·5	41·6	30·7
		" 2·5	42·4	29·7
B	K	None	18·5	12
	K, Na	None	34	16
	K, Na	Ganglioside 0·3	18·4	14
C	K, Na	None	34·4	17·4
		Protamine sulphate, 0·5	33·3	16·4

All reaction mixtures contained 125 mM-tris Cl, pH 7·4, 3 mM-$MgCl_2$, and 3 mM-tris adenosine triphosphate; KCl when added was 30 mM and NaCl, 200 mM. The tissue fractions of Expt. A were used 2 days after preparation, of Expt. B, 3 days after preparation and of Expt. C, 8 days after preparation. Ganglioside preparation A was used.

examining tissue fractions which had been kept at 0° for different periods of time, susceptibility to gangliosides, like activation by Na, was greater in an older preparation (Fig. 5). Clupein, the basic protein which undergoes several interactions with gangliosides and with the microsomal fraction, brought about noticeable aggregation of these particles in the experiments of Table 3, but nevertheless caused no loss but some increase in their adenosine triphosphatase activity.

DISCUSSION

The sodium-activated adenosine triphosphatases previously observed in subcellular fractions from neural tissues are not of high potency when their rates of reaction are referred to the weight of tissue from which they are derived. SKOU's (1957) preparation from crab nerve liberated about 20 μg of phosphate in 30 min with

material from 20 mg of tissue, corresponding to about 60 μmoles/g tissue/hr. That of JÄRNEFELT (1961) from rat brain liberated 0·75 μmoles phosphate in 10 min with material from 1/50 of a rat brain; taking the whole rat brain weight as 3·5 g, the corresponding rate is again about 60 μmoles/g tissue/hr. The similarity of these values contrasts with a general impression of greater metabolic rate in the mammalian and in

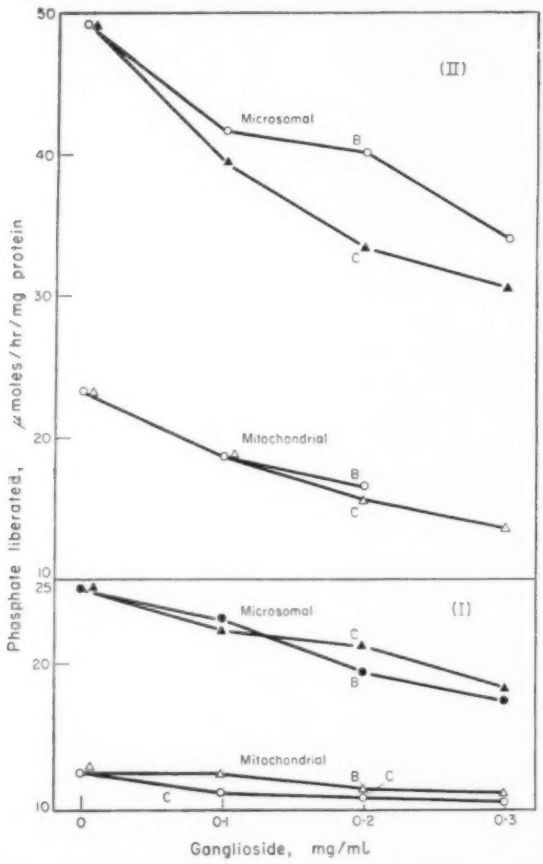

FIG. 5.—Effect of ganglioside preparations B and C on adenosine triphosphatases in the presence of 3 mM-ATP, 3 mM-$MgCl_2$, 30 mM-KCl, 200 mM-NaCl and particulate preparations kept (I) 1 day and (II) 4 days at 0–4°.

the cerebral tissue. In the case of the brain, the rate would suggest an adenosine triphosphatase activity which was a small fraction only of that of the whole tissue, for GORE (1951) observed liberation of inorganic phosphate from adenosine triphosphate by suspensions of guinea pig brain to proceed at 2040 μmoles/g tissue/hr. Moreover, in studying a fraction activated 30 per cent by Na salts, JÄRNEFELT (1961) did not appear to have been employing the conditions or the cerebral fraction most sensitive to Na, for in unfractionated rat-brain suspension, HESS and POPE (1957) had observed 60 to 100 per cent activation by Na^+. PALLADIN (1961) recorded a cerebral adenosine triphosphatase activity of 650 μmoles phosphate/g tissue/hr, but did not report Na^+ activation.

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Present investigations come much closer to studying a major, Na-sensitive cerebral adenosine triphosphatase. In relation to the weight of tissue, the precipitate from centrifugation at 10^7 **g**-min has 10 times the activity of that previously observed, and the proportional increase in activity caused by Na can rise to a value 5 times greater than previously observed. The rate thus approaches that likely for the formation and utilization of energy-rich phosphate in a tissue whose respiratory rate of 120 μmoles O_2/g/hr probably implies a phosphate turnover of some 720 μmoles/g tissue/hr. Again, the Na-activated enzyme has been found in a so-called microsomal fraction which is difficult to deposit; the likelihood that this carries a distinct enzyme has been increased by observing a characteristic difference in pH optima between its activity and that of the mitochondrial fraction. (The pH curves of Fig. 1 suggest in addition that the mitochondrial fraction may contain a small proportion of the microsomal enzyme.) Reasons for the greater activity found in the microsomal fraction of the present investigation may relate to the manner of homogenizing which was advocated by EMANUEL and CHAIKOFF (1957) and also to our inclusion of the material which proved more difficult to deposit. Its greater sensitivity to Na may depend on these factors or on the changes occurring by keeping it at 0°.

Material deposited at 10^7 **g**-min has been found to be richest in ganglioside-like material and to be of greatest potency in combining with basic proteins (WOLFE, 1961; WOLFE and MCILWAIN, 1961). Both these agents modify cation transport in cerebral tissues (MCILWAIN, WOODMAN and CUMMINS, 1961) in which the Na-sensitive adenosine triphosphatase is suspected to be involved. A mechanism has been proposed for the action of such a membrane-sited adenosine triphosphatase (MCILWAIN, 1961*b*, *c*). The inhibitory action of suramin appears less specific; this substance has been noted to inhibit certain other enzymes (WILLS and WORMALL, 1950) including phosphatases (BELING and ZICZFALUSY, 1959) and in the present study, it inhibited the adenosine triphosphatase activities of both mitochondrial and microsomal fractions. It is to be noted that, although Na and K requirements suggest the possibility of connecting the microsomal adenosine triphosphatase activity with the performance of osmotic work, a major activity of microsomal fractions of other tissues has been concluded to be protein synthesis, and that under certain situations synthetic systems utilizing adenosine triphosphate might behave as adenosine triphosphatases. These possibilities are open to investigation by subfractionating and examining further the present microsomal preparation, and such studies are in progress.

SUMMARY

Guinea pig cerebral cortex disintegrated in sucrose solutions was differentially centrifuged to yield 5 fractions. The activities of the fractions in catalysing liberation of inorganic phosphate from adenosine triphosphate was measured in the presence of a number of added substances.

With Mg, K and Na ions the unfractionated suspension liberated phosphate at about 2000 μmoles/g cortex/hr; a nuclear fraction at 290, a mitochondrial fraction at 350, a mitochondrial plus microsomal fraction at 490, a microsomal fraction at 590 and a supernatant at 150 μmoles/hr with material derived from a gram of cortex.

Liberation by the mitochondrial fraction was maximal at pH 9 and by the microsomal at 7·5. In media containing Mg and K ions, Na^+ accelerated the activity of the microsomal fraction to a much greater extent than it did that of the mitochondrial

fraction. Depending on K^+ concentration and other factors, activation of the mitochondrial fraction was increased by 0–30% and the microsomal fraction by 30–170%.

The adenosine triphosphatases were relatively insensitive to simple anions, but were inhibited by preparations of cerebral gangliosides and of the trypanocidal agent suramin.

Acknowledgement—We are grateful to Dr. J. JÄRNEFELT for informing us of his findings before they were published.

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MEMBRANE POTENTIALS IN GUINEA-PIG CEREBRAL CORTEX SLICES *IN VITRO*. THEIR DEPENDENCE ON SUBSTRATE AND OXYGEN. THE EFFECT OF CLUPEIN AND OF GANGLIOSIDE PREPARATIONS

H. H. HILLMAN

Department of Biochemistry, Institute of Psychiatry, (British Postgraduate Medical Federation, University of London) Maudsley Hospital, London, S.E.5

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PREVIOUSLY it was shown that cellular elements in isolated mammalian cerebral tissue slices *in vitro* become polarized after incubation, and that this level of polarization is dependent upon their ionic environment (LI and MCILWAIN, 1957; HILLMAN and MCILWAIN, 1961). The dependence of this polarization on the substrate and presence of oxygen, and the effects of clupein and ganglioside preparations, and their interaction, are examined in the experiments reported here; these latter substances were examined in view of their effects on the metabolic response of the tissue to electrical stimulation (THOMSON and MCILWAIN, 1961).

METHODS

The technique of preparation and recording was reported previously (HILLMAN and MCILWAIN, 1961). Outer slices of guinea pig cerebral cortex weighing 50–100 mg were cut 0·30–0·35 mm thick; the tissue chamber contained 10 ml of incubation fluid of which 3 ml were usually in contact with the tissue at any one time. Resting membrane potentials were measured using micro-electrodes filled with 2·7 M–KCl, and having tips less than 1 μ and resistances of 10–50 mΩ. The membrane potentials studied were the sudden, maximal negative potentials recorded in a complete penetration of the slice after it had been incubated at 37° in a Krebs–Ringer bicarbonate buffered solution for 35–40 min. In the experiments on hypoxia, and in those using clupein and ganglioside preparations, effects could be studied either from the beginning of incubation, or after equilibrium had been reached in oxygenated Krebs–Ringer bicarbonate glucose saline, and 25 control readings had been recorded; in the latter case, a second control series was taken after return to the control medium. After any change of solution, 20 min were allowed to elapse before recording was re-commenced so that equilibration of the tissue with the medium could take place.

Hypoxia was induced by changing the 5% CO_2-in-O_2 for a 5% CO_2-in-N_2 cylinder. Due to the flow of air into the incubating chamber via the hole through which the micro-electrode passed, anoxia was not achieved, the gas phase above the slice having a composition O_2:CO_2:N_2 of 10:4:86 compared with 70:4:26 during oxygenation; (these concentrations were measured on a Scholander apparatus). The gas-flow rate in both cases was 100 ml/min.

When a penetration was made through the thickness of the slice without recording any potential, a 'zero' reading was noted. For discussion of its significance, see HILLMAN and MCILWAIN (1961) and HAAPANEN, KOLMODIN and SKOGLUND (1958)

Clupein sulphate (L. Light & Co.) was used, and the ganglioside was kindly prepared by Dr. D. B. GAMMACK and Mr. D. A. BOOTH by the method of MCILWAIN (1961).

RESULTS

It can be seen (Table 1) that resting membrane potentials in cerebral slices *in vitro* could be maintained in 10 mM-pyruvate, lactate or oxaloacetate at levels which were

not significantly lower than in glucose substrate. With 10 mM-L-glutamate, however, the resting potential was significantly lower than that with glucose, and not significantly different from the potentials recorded in the absence of added substrate.

Hypoxia caused a lowering of the resting potential (Table 2) which was even more marked than in the absence of substrate (Table 1). This was shown in both experimental series, whether hypoxia was induced from the beginning of incubation or

TABLE 1.—GUINEA-PIG CEREBRAL CORTEX SLICES *in vitro*. MAINTENANCE OF MEMBRANE POTENTIAL BY DIFFERENT SUBSTRATES

Substrate (10 mM)	Membrane potential (mv, mean ± S.D.)	Substrate (10 mM)	Membrane potential (mv, mean ± S.D.)
Glucose*	58 ± 12 (269)	Oxaloacetate	54 ± 10 (100)
Pyruvate	58 ± 12 (100)	L-Glutamate	43·6 ± 6 (75)
Lactate	54 ± 11 (125)	No substrate	45 ± 13 (100)

Krebs-Ringer bicarbonate-buffered saline.
Figures in brackets are number of readings.
* Figure from HILLMAN and MCILWAIN (1960) for comparison.

TABLE 2.—EFFECT OF HYPOXIA ON MEMBRANE POTENTIALS IN GUINEA-PIG CEREBRAL CORTEX SLICES *in vitro*

Condition	Membrane potential (mv, mean ± S.D.)	Zero* readings	Condition	Membrane potential (mv, mean ± S.D.)	Zero* readings
Oxygen	53 ± 12 (75)	3			
Hypoxia	25 ± 11 (45)	73	Hypoxia	36 ± 11 (80)	14
Oxygen	42 ± 11 (60)	48	Oxygen	43 ± 11 (80)	5

Left hand column, incubated in oxygen (3 expts.); right hand column, incubated in hypoxia (3 expts.).
Figures in brackets are number of readings.
* See Methods for explanation.

whether it was induced after a state of equilibrium had been reached during incubation in an oxygenated medium in an oxygen gas phase (see Methods). In neither case was recovery complete when oxygenated conditions were restored.

When slices were incubated under hypoxic conditions and in the absence of substrate (3 experiments of 50 penetrations each) the cells would not polarize; even if the slices were subsequently oxygenated and glucose added to make the solution 10 mM no potential could be recorded. If the slices were incubated without substrate, low scattered readings were recorded; when the tissue was then rendered hypoxic, the cells depolarized completely, but not irreversibly (Fig. 1).

In 4 experiments tissue was incubated in normal Krebs–Ringer bicarbonate glucose saline, then 0·3 mg clupein/ml saline was added; there was no significant change in the resting potentials observed (Table 3). If on the other hand, clupein was present in the same concentration from the beginning of the experiment, either the cells would not polarize at all, even after incubation for 90 min, or they became polarized at low levels—39–46 mv. After a 20-min interval a further 25 readings were made but the levels did not increase during this successive period of incubation with clupein (Table 4).

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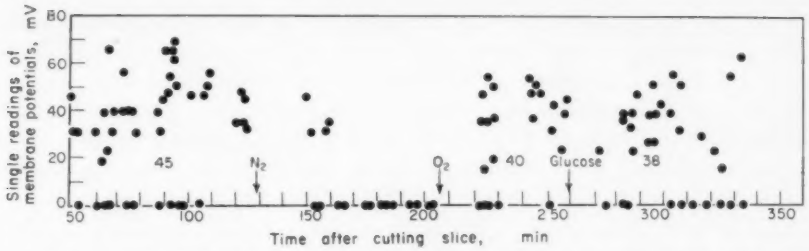

FIG. 1.—Individual readings of membrane potentials in guinea-pig cerebral cortex slices incubated in the absence of substrate. Recording was started 50 min after cutting slice. At first arrow tissue subject to hypoxia; at second arrow oxygen supplied to tissue; at third arrow solution made 10 mM in glucose. Number in each condition, mean of readings. Hiatuses give the time for tissue to equilibrate, during which no recording was attempted. Note (i) low readings with wide scatter in absence of substrate (ii) large number of zero readings (iii) reversible depolarization by hypoxia (iv) absence of increase of potential on adding substrate (v) ability of some cells to maintain membrane potentials for 4 hr in the absence of added substrate.

TABLE 3.—EFFECT OF CLUPEIN SULPHATE AND GANGLIOSIDE PREPARATION ON MEMBRANE IN GUINEA-PIG CEREBRAL CORTEX SLICES *in vitro*

Condition	Membrane potential (mv, mean ± S.D.)	Condition	Membrane potential (mv, mean ± S.D.)
Control	53 ± 12 (100)	Control	56 ± 9 (125)
Clupein	50 ± 12 (100)	Ganglioside	58 ± 9 (125)
Control	51·5 ± 12·5 (100)	Control	60 ± 10 (75)

Clupein sulphate (0·3 mg) and ganglioside preparation (0·3mg.) were added per ml medium.
Left hand column, 4 expts; right hand column, 5 expts.
Figures in brackets are number of readings.

TABLE 4.—EFFECT ON MEMBRANE POTENTIALS OF GUINEA-PIG CEREBRAL CORTEX SLICES *in vitro* OF (A) INCUBATING WITH CLUPEIN DURING TWO SUCCESSIVE PERIODS and (B) ADDING GANGLIOSIDE PREPARATION DURING SECOND CLUPEIN PERIOD

	Clupein (1st period) (mv, mean ± S.D.)	Clupein (2nd period) (mv, mean ± S.D.)	Ganglioside (mv, mean ± S.D.)
(A)	39 ± 13	45 ± 16	—
	43 ± 10	46 ± 10·	—
	Depolarized	Depolarized	30 ± 13
	Depolarized	Depolarized	32 ± 15

	Clupein (mv, mean ± S.D.)	Ganglioside (mv, mean ± S.D.)
(B)	48 ± 7	58 ± 10
	43 ± 11	53 ± 8
	41 ± 11	46 ± 10
	Depolarized	48 ± 13
	Depolarized	36 ± 11
	Depolarized	37 ± 12

Clupein sulphate (0·3 mg) and ganglioside preparation (0·3 mg) were added per ml medium.
Each figure is from 25 readings, and each horizontal line one experiment.
Each period lasted 40–50 min.

If during this second period 0·3 mg of a ganglioside preparation/ml of medium was added, there was an increase in the resting potential; this was significant ($P < 0{\cdot}01$) in 5 out of 6 experiments. In 3 experiments the elements, which had not been polarized, became polarized to mean levels between 37 and 48 mv, while in 3 experiments where the cells were polarized in clupein to mean values of 41–48 mv, they became more highly polarized to levels of 46–58 mv (Table 4). Thus ganglioside appeared to restore the level of polarization of cells hypopolarized by clupein.

Ganglioside itself when added to tissue incubated in normal solution produced a slight but not significant rise in the resting potential in 5 experiments (Table 3); after return to the normal medium, this rise was maintained in 3 experiments.

DISCUSSION

Pyruvate, lactate and oxaloacetate substrates can support the resting potential at a level not significantly lower than can glucose. With L-glutamate, hypoxia or lack of substrate polarization is at a much lower level. These findings are in parallel with the observations of WOODMAN and MCILWAIN (1961) that the former four substrates allow substantial phosphocreatine resynthesis during incubation, while under the latter three conditions the rate of resynthesis is kept at a lower level. This would suggest a correlation between the cells' ability to polarize their membranes and their energy metabolism. CUMMINS and MCILWAIN (1961) have computed that during electrical stimulation of cerebral tissue slices, 18 per cent of the energy liberated by the breakdown of phosphocreatine can be accounted for by the increased work done during the enhanced efflux of potassium. If this energy source is depleted, it might be expected that less energy would be available to maintain the potassium gradient across the membrane, so closely related to the resting potential (HILLMAN and MCILWAIN, 1961).

The depression of resting potential by hypoxia and lack of substrate has previously been demonstrated by LI and MCILWAIN (1957); KOLMODIN and SKOGLUND (1959) reported a small rapid fall in the resting potential of cat spinal motoneurones *in vivo* under hypoxic conditions, followed by a slower but greater fall. In the present experiments, 20 min elapsed before the effect of hypoxia was examined, and it is probable that the observations seen here correspond to the slower change they observed. The fact that the average level of potentials recorded here fell indicates that most cells were hypopolarized by hypoxia, but the increase of zero readings—largely reversed when the tissue was restored to oxygen—suggests that some cells in the slice are much more sensitive to hypoxia than others; they depolarize completely, while others only hypopolarize. The fact that following the tissue's oxygenation after hypoxia there are more zero readings than before hypoxia suggests that this procedure permanently depolarized some cellular elements.

Clupein sulphate appears to prevent cellular repolarization immediately after tissue excision, but to have no significant effect once the cellular elements have been allowed to repolarize. Immediately after excision, hypoxia in the absence of substrate irreversibly prevents cellular repolarization, even if oxygen and glucose are subsequently supplied. However, tissue hypopolarized by the absence of substrate early in incubation, though completly depolarized by superimposed hypoxia, retains its ability to repolarize when subsequently reoxygenated. These are two examples of conditions in which the membranes are more sensitive to hypopolarizing agents immediately

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after tissue excision than they are once equilibrium has been reached. During the first 20–30 min, potassium is being reconcentrated in the cells (KREBS, EGGLESTON and TERNER, 1951; PAPPIUS and ELLIOTT, 1956) and phosphocreatine is being resynthesised (MCILWAIN, 1952).

The repolarization by ganglioside preparations of cellular elements hypopolarized by clupein is parallelled by the findings of THOMSON and MCILWAIN (1961) of the restoration by gangliosides of the metabolic response of cerebral slices to electrical stimulation after this had been prevented by clupein, and supports their assertion that these substances act on the membrane. They appear to have more effect on the cell membrane when the ion flux is enhanced as during early incubation or electrical stimulation, and it might be expected that they would cause profound effects during the rapid ion movements of the action potential *in vivo*.

SUMMARY

Resting membrane potentials in guinea-pig cerebral cortex slices *in vitro* in Krebs–Ringer bicarbonate solution with 10 mM-pyruvate, lactate or oxaloacetate as sole substrate are at the same level as with glucose as substrate.

They are lower in the absence of substrate, with 10 mM-L-glutamate as sole substrate, or during hypoxia. The membranes are depolarized by hypoxia in the absence of substrate.

The lowering of the resting potential induced by addition of 0·3 mg clupein/ml medium is counteracted by addition of 0·3 mg ganglioside preparation/ml medium.

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FLUID CONTENT AND COMPARTMENTS IN ISOLATED CEREBRAL TISSUES

S. VARON and H. MCILWAIN
Department of Biochemistry, Institute of Psychiatry,
(British Postgraduate Medical Federation, University of London)
Maudsley Hospital, S.E.5

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ION MOVEMENTS are fundamental to neural activity, and their study in cerebral tissues (CUMMINS and MCILWAIN, 1961) has prompted the following re-examination of tissue fluids. This is because, although potassium loss and assimilation can be investigated with minimal knowledge of concomitant changes in fluids, the fluid movements must be known in order to appraise changes in sodium and chloride ions. In addition, some theories of ion movement (MILLER, 1960) propose that the primary point at which metabolically derived energy is applied in performing osmotic work, is on fluid rather than on ions.

The present study involved appraising: (1) Changes occurring during the initial contact of the cut tissue with fluids to be used in incubation. Changes of this category have received relatively little attention, but their study is made more feasible by the recently developed technique of slicing without contact with fluid, using a narrow blade mounted in a bow-cutter (RODNIGHT and MCILWAIN, 1954; MCILWAIN, 1961). (2) Change during subsequent incubation has been examined by measuring total fluid, and also the inulin content of the tissue after its incubation in inulin-containing media. The use of inulin to obtain a measure of intra- and extra-cellular spaces in cerebral tissue was based on the studies of KOREY and MITCHELL (1951), ALLEN (1955), PAPPIUS and ELLIOTT (1956) and MCLENNAN (1957) and is appraised further below. (3) The methods developed have been applied to determine fluid changes during electrical excitation of the tissue, and during the action of basic proteins: both these agents were found by THOMSON and MCILWAIN (1961) to alter tissue weight, presumably by fluid changes.

EXPERIMENTAL

Media and tissue

Glycylglycine saline contained 124 mM-NaCl, 5 mM-KCl, 1·24 mM-KH_2PO_4, 1·3 mM-$MgSO_4$, 2·8 mM-$CaCl_2$, 10 mM-glucose, and 30 mM-glycylglycine taken to pH 7·4 with M-NaOH and was saturated with O_2. Bicarbonate saline, unless otherwise specified, differed only in containing 26 mM-$NaHCO_3$ in place of glycylglycine and was equilibrated with 95% O_2:5% CO_2. In media which contained inulin (see below) its concentration was 1% unless stated otherwise.

Adult guinea pigs of either sex, weighing 250–400 g were stunned with a blow on the neck and exsanguinated by cutting the neck; the brain was removed as described by MCILWAIN and RODNIGHT (1962) within 1·5 min of stunning. A hemisphere was taken, subcortical structures removed, and it was placed on a cutting table (MCILWAIN, 1961) with the cortex uppermost. From this, three successive slices 0·35 mm thick were cut at room temperature (17–20°) in one of two ways. (1) With a bow cutter and glass guide (MCILWAIN, 1961), avoiding all contact with fluid. The slices were picked up with a bent wire rider of a torsion balance and weighed to the nearest mg; this is the method used in most experiments. (2) With a blade and the same glass guide, lubricated with one of the incubating media described above; after cutting, these slices were floated from the blade or guide into a shallow dish of

the same medium. From this they were picked up on the rider and were usually drained free from fluid (see below) before weighing.

Dry weight. Slices already weighed on a torsion balance were placed in weighed crucibles and reweighed after 90 min at 105°. A further 30 min at 105° caused no further change.

Incubation and extraction. (a) When manometric apparatus was used, weighed slices of 50–100 mg were placed in 3·5 ml of medium in a conventional Warburg vessel. With bicarbonate medium, the centre wells were empty and 95% O_2:5% CO_2 was used in the gas phase; with glycylglycine saline, the centre wells contained 0·12 ml of 5 N-NaOH and the gas was O_2. After a measured period at room temperature, the flasks were transferred to the 37° thermostat for a further timed period, after which a flask was removed, its slice picked out with a wire rider, drained, weighed, and ground in 4 ml of 6% trichloroacetic acid with a homogenizer pestle which fitted a 10 ml centrifuge tube. The other slices were removed similarly. The tubes were spun at 800 **g** for 10 min and the supernatant fluids poured into 25 ml measuring flasks. The residues were resuspended in 5 ml of water or 6% trichloroacetic acid, centrifuged again and the supernatants added to their respective flasks. A further extraction yielded no more inulin. (b) When electrical pulses were applied, the quick-transfer electrodes of McIlwain (1961) and McIlwain and Rodnight (1962) were used. Slices floating in one of the salines were taken up by the lower jaw of the electrodes, the holder was closed and transferred with the slice to 5 ml of already oxygenated medium in a 20 ml beaker which the holder fitted. The assembly was placed at 37°, the period spent by the slice in fluid at room temperature being noted. Electrical pulses were supplied at 100 per sec by the apparatus described by McIlwain (1961); they were of exponential time-voltage relationship, alternating, of peak potential 10 v and time-constant 0·4 msec; for choice of these characteristics see McIlwain (1954). Slices were released at defined times, usually into a fluid at 0°, and were then taken up by a wire rider, drained free from fluid, weighed and extracted as in (a) above.

Inulin determination

The reaction with resorcinol employed by Hubbard and Loomis (1942), Roe, Epstein and Goldstein (1949) and Preedy (1954) has been examined systematically in relation to its use for determining inulin in the present experiments, and as a result the following procedure is recommended.

Procedure. Glass-stoppered (C14) tubes of 15 ml were used in a rack taking 36 tubes and enabling them to be transferred together from a cold bath of running tap water to a bath thermostatically controlled at 80 ± 0·15°. Immersion of the rack and tubes caused the hot bath to fall by 4° and this was made good within 1 min by a current of steam. The cold bath was fitted with a lid to exclude light. A resorcinol reagent was made by dissolving 0·1 g resorcinol and 0·25 g thiourea in 100 ml glacial acetic acid and was kept in a dark bottle; a standard solution of inulin (1 mg/ml) was kept in a refrigerator. To the test tubes of the rack in the cold bath were pipetted 2 ml of either tissue extracts, or standard inulin solutions (5–30 μg). The resorcinol reagent (1 ml) and 0·46 mM-$FeCl_3$ in conc. HCl (7 ml) were added, the solutions were mixed and the tubes put at 80° for 15 min. They were removed to the cold bath and their optical densities were read at 514 mμ, between 5 and 60 min after cooling. During the reading of a large batch of tubes, only a few were taken from the cold bath at a time, to minimize exposure to light.

Choice of conditions. Procedures with and without $FeCl_3$, thiourea and acetic acid are described. In exploratory experiments under different conditions, the light absorption of solutions resulting from the treatment of 20 μg inulin were read at each 2–10 mμ from 360–540 mμ, and usually showed peak absorption or inflexions at 395–410 mμ (sometimes complex), at 460–490 mμ, and at 510–520 mμ. In the absence of $FeCl_3$, the peak around 400 mμ was most prominent and the absorptions were not greatly affected by thiourea. Addition of $FeCl_3$, with or without thiourea, was examined at concentrations of 0·0046, 0·046, 0·46 and 4·6 mM. These additions enhanced absorption a little (from 0·18 to 0·2) around 400 mμ, and greatly increased it at 460–540 mμ; the two peaks in this region became quite distinct. The greatest effect was found at 514 mμ and with $FeCl_3$ at 0·46 mM, when the optical density increased from 0·11 to 0·24. When a narrower range of $FeCl_3$ concentrations around 0·46 mM was investigated, the readings at 514 mμ were found to be less dependent on $FeCl_3$ concentration than were the other peaks, and reading at 514 mμ was therefore chosen in the standard procedure. With this concentration of $FeCl_3$, thiourea proved advantageous and linear curves were obtained between 2·5 and 60 μg of inulin; at 80 μg, readings were 3 per cent below those expected from strictly linear relationships.

Study of time relationships of different stages in the procedure showed that before heating, the order or time of addition was not exacting and that tubes could be left for 40 min in the cold bath without effect on the final colour developed. Time and temperature of heating were important in relation to the subsequent stability of the colour after the tubes were cooled: at 80°, heating for less than 15 min (10 or 12 min) gave solutions whose optical density continued to increase after cooling; if heating were continued for 20 or 25 min, the colour faded. Heating for 15 min was adopted; it gave readings 15–20 per cent greater than did 10 or 20 min heating. Temperature of heating was examined while otherwise maintaining the conditions of the standard procedure; variation from 78° to 82° was found to be tolerated; colour intensity and stability were lower at 75° or 85°.

Reproducibility and specificity. Standard inulin solutions run in duplicate or triplicate on many different days over a period of some months gave the following optical densities (mean ± S.D., 20 observations each): 5 μg inulin, 0·061 ± 0·002; 10 μg, 0·123 ± 0·004; 20 μg, 0·239 ± 0·007. Inulin was normally included in incubation media at 1%, and the media were diluted 500 times to give about 20 μg/ml. Under these conditions the media, either glycylglycine- or bicarbonate-buffered, alone or with additions of protamine or gangliosides, gave no apparent inulin when none was added, and did not affect the readings from standard inulin solutions. Glucose at higher concentrations gave the same colour reaction as did inulin. By the standard procedure, an optical density–wavelength curve closely similar to that of inulin was found, glucose giving readings corresponding to 2·2 per cent of its weight of inulin. With tissues after incubation, the extraction procedure described above gave their inulin in a solution containing 1% trichloroacetic acid; this and greater concentrations of the acid were found not to affect colour development in the standard procedure (with reagents lacking thiourea and acetic acid, interference was observed). The slices themselves, without added inulin, gave a small and consistent apparent inulin content corresponding to an optical density of 0·006 ± 0·001 (mean ± S.D., 18 values) per 100 mg of tissue. This tissue blank has been subtracted from all values reported; its magnitude was found to be unaffected by addition of known quantities of inulin, protamine or gangliosides.

Expression of results. The density of tissue and fluids is taken as 1, and the inulin is assumed to be at uniform concentration in any fluid to which it has access. The inulin space (i.s.) is then expressed as μl occupied/100 mg fresh wt of tissue, by dividing (μg inulin found per 100 mg tissue) by (μg inulin found per μl fluid).

$$\text{i.s.} = \frac{\mu\text{g inulin found per 100 mg tissue}}{\mu\text{g inulin found per ml fluid}}$$

The 100 mg of fresh tissue contains 80 mg of water and 20 mg of solids, but additional fluid (a.f.) becomes associated with it during incubation. The space not occupied by inulin after incubation of 100 mg fresh tissue is then taken as (80 + a.f. − i.s.). Comment is made in the Discussion on the validity of these assumptions.

RESULTS

In preparation for metabolic experiments, tissue slices are usually placed in incubating media first at room temperature (perhaps only for brief periods) and subsequently at 37°. Fluid changes during these two stages have been examined separately.

Observations at room temperature

Brief dipping and draining. It is necessary to know how completely a slice can be freed from adherent fluid. The procedure found most suitable for this was to place a bent wire below a slice floating fully extended in fluid, and to transfer the slice with the wire to a clean, dry glass surface. With the slice still hooked to the wire first one and then the other side of the folded slice was laid flat on the glass, remaining in contact for about 1 sec each time. Slices of 50 to 80 mg which had been cut dry were placed in glycylglycine saline for 10 sec and drained in this way. Their weight became constant after contact for 6 to 8 times, at a value 4 to 8 mg greater than their original weight; mean increase ± S.D., 4·6 ± 1·15 mg (31 values); mean percentage increase ± S.D., 8·5 ± 2·4 (31 values). This was adopted as standard draining procedure.

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Subsequently contact diminished the weight by less than 1 mg and left a cloudy streak on the glass, suggesting that material other than fluid was being removed. When drained slices were replaced in fluid for 10 sec and draining repeated the same drained weight was found. Because of this behaviour more complete draining, though possible, has not been adopted; the value of 8·5 per cent appears as a zero-time value where appropriate, e.g. in Fig. 1, and is referred to as 'adhering medium'.

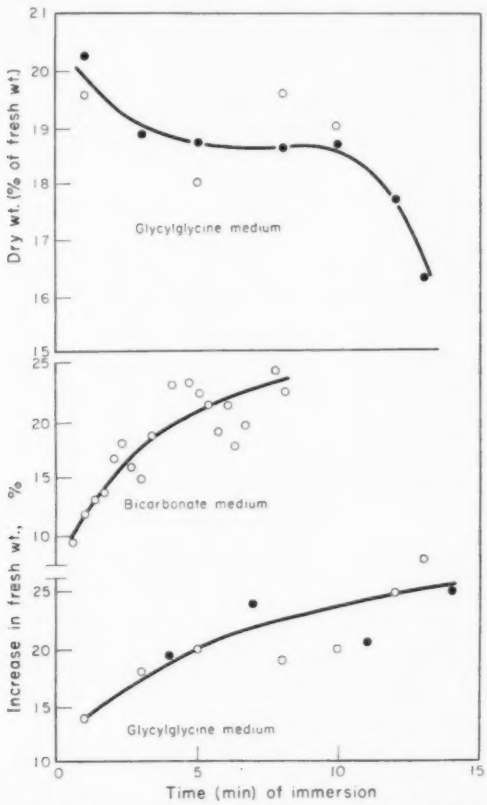

FIG. 1.—Change in weight in slices of guinea pig cerebral cortex during immersion in oxygenated media at 17–20°. Points ○: individual slices; ●: mean values for groups of 3 slices, 1 representing each of the 3 depths in the cortex normally sampled.

Immersion. Slices which had been cut dry were immersed in media for periods up to 20 min, before draining and reweighing as described above. Results (Fig. 1) show a progressive increase in weight over the first 4 to 5 min in both glycylglycine- and bicarbonate-buffered media. Slices cut first from the outer convexity of the brain did not differ markedly from the second or third slices cut at increasing depths in the cortex. Between 10 and 20 min after immersion in glycylglycine media, slice weight continued to increase but change during this period was not followed in detail as it is not involved in typical metabolic experiments. The dry weight of the tissues, by contrast, fell gradually during the first 8 min of immersion in both bicarbonate and glycylglycine salines. Some 1 per cent of the original 19·6 $\pm$ 0·8 per cent (18 values) of dry matter

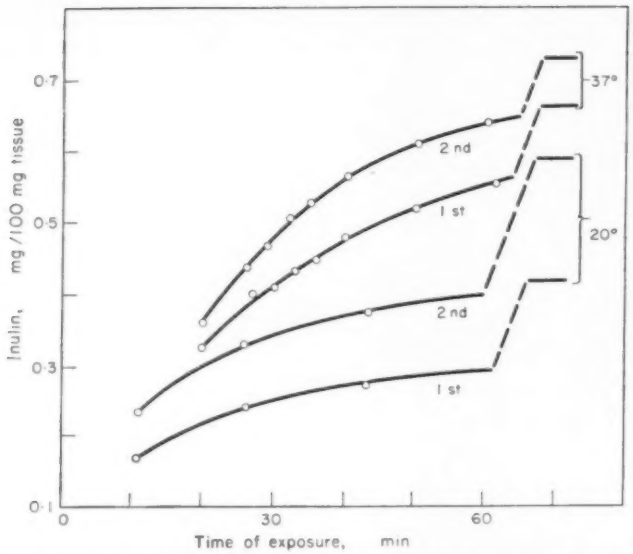

FIG. 2.—Release of inulin from slices of guinea pig cerebral cortex. The slices had been prepared and incubated as described in Table 1, in bicarbonate media with 1% of inulin. They were then exposed, in fresh media without inulin, agitated by the oxygenating gas at the two temperatures indicated. Samples of the media were taken at intervals, and inulin was determined in these and in the tissues at the end of the experiment. Slices are designated as being the *first* cut from the outer convexity of the cortex, or the *second* cut immediately below, and their total inulin on transfer to the second medium, computed from the determinations described, is indicated by the horizontal lines.

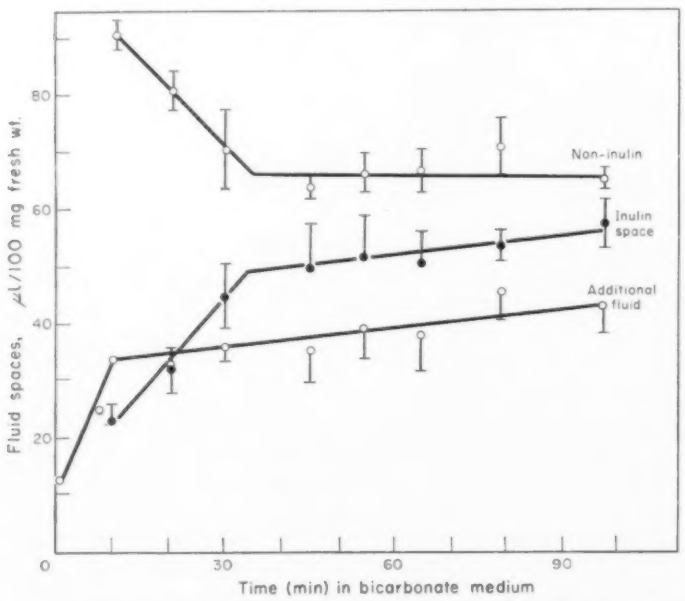

FIG. 3.—Fluid changes on preparing and incubating cerebral cortical slices in glucose bicarbonate media. Points give mean values (4 values) and vertical lines indicate S.D.

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was lost in this time (Fig. 1). After 10 min, a much more considerable loss in solids was observed, some 4 per cent being lost to glycylglycine saline between 10 and 15 min.

Incubated tissues

Inulin. Two types of experiments were carried out to validate the use of inulin as a measure of extracellular space. (1) Slices cut dry were incubated for 60 min in glycylglycine media with either 1% or 0·25% of inulin. Essentially the same measure of inulin space was obtained (Table 1). (ii) Slices were incubated first in glycylglycine

TABLE 1.—TISSUE INCUBATED WITH DIFFERENT INULIN CONCENTRATIONS AND DIFFERENT BUFFERS

Slices were cut dry, weighed, and placed in media in Warburg flasks; these processes involved the tissue being in fluid for 10·5–13 min. Oxygen or O_2–CO_2 was passed for 10–14 min and the vessels were incubated for 60 min, after which the tissue was drained, reweighed and placed in trichloroacetic acid. Mean values are followed by S.D. and (in parentheses) number of observations.

Measurement (per unit fresh wt)	*A*: Glycylglycine medium with Inulin (mg/ml) 2·5	10	*B*: Inulin (10 mg/ml) in Glycylglycine medium	Bicarbonate medium
Respiration (μmoles O_2/g/hr)	97·2 ± 8·8 (11)	99 ± 12 (6)	99 ± 12 (6)	—
Increase in wt (mg/100 mg)	37·6 ± 4·5 (12)	37·6 ± 5·4 (12)	38·6 ± 4·3 (6)	36·6 ± 6·7 (6)
Inulin (μl occupied/100 mg)	60·2 ± 9·2 (12)	62·3 ± 6·4 (12)	62·5 ± 5·7 (6)	62·2 ± 7·6 (6)
Volume not occupied by inulin (μl/100 mg)	57·3 ± 6·9 (12)	55·3 ± 4·2 (12)	56·1 ± 4·3 (6)	54·4 ± 4·3 (6)

medium with 1% inulin for 60 min, then drained, reweighed and transferred to 10 ml of inulin-free medium. The fluid was sampled at intervals to show the release of inulin (Fig. 2). After 60 min at 37°, some 90 per cent of the inulin had been released, and release was still in progress. It was concluded that no firm binding took place between inulin and the tissue. Release was slower at room temperature; both results are consistent with diffusion being the process concerned.

The process of uptake of inulin by the tissue is shown in Fig. 3, together with concomitant changes in weight. Inulin uptake clearly shows two phases of which the second (30 min to the end of the experiment) runs parallel to the change in weight: both are regarded as measures of the amount of fluid which is becoming associated with the tissue on incubation. The initial period of inulin uptake (10 min at room temperature followed by 20 min at 37°) is therefore considered to indicate equilibrium of inulin from the medium with the available extracellular spaces of the tissue. When the space not occupied by inulin is calulated on the basis of the initial volume of tissue it is seen (Fig. 3) to be approximately constant during the second phase of inulin uptake. A number of the following comparisons have been made after 60 min incubation of the tissue, when it is seen (Fig. 3) that it is in equilibrium with inulin.

Prior exposure at room temperature; buffers. Uptake of fluid and of inulin by the tissue were almost identical in glycylglycine- and in bicarbonate-buffered media (Table 1). However, in any one buffer the subsequent fluid relationships of slices were found to be markedly dependent on the time spent in handling them in fluids before good metabolic conditions at 37° in 95-100% O_2 were established (Fig. 4). Even brief exposure ($\frac{1}{4}$–2 min) at 37° in air rather than oxygen, resulted in much additional fluid uptake after the subsequent incubation. At room temperature, the change which affected fluid uptake was accomplished more slowly, needing 7–8 min to reach the

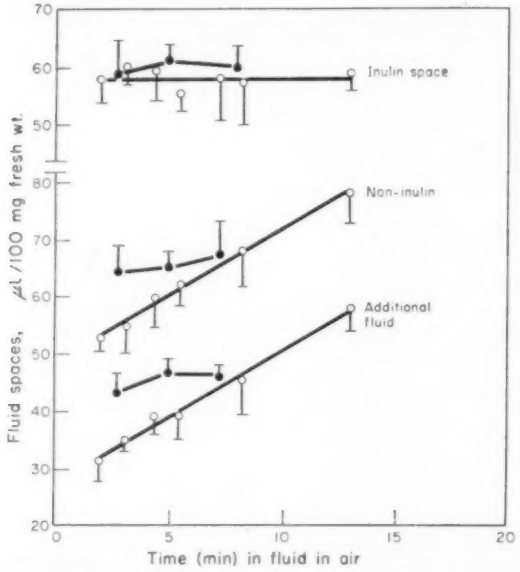

FIG. 4.—Fluid changes in cerebral cortical slices in glycyl glycine-glucose media with inulin, as a result of exposure in air (not oxygen) for the period indicated; all tissues were subsequently incubated for 60 min at 37° in oxygen. After incubation they were drained, weighed and their inulin content determined. Points ●: in air at 37°; ○, in air at 17–21°; mean values with S.D. indicated by vertical lines.

45 per cent found within 2 min at 37°. These changes proved to be almost entirely in the non-inulin space of the tissue, the inulin space remaining almost constant.

Zonal differences. The slicing procedure employed (see Experimental section) yielded successive slices from one guinea pig hemisphere which represented the cortex at depths of: first, 0–0·35 mm; second, 0·35–0·7 mm; third, 0·7–1·05 mm. These have been found to differ in their swelling, measured by inulin uptake in experiments such as those of Fig. 3 or 4. In a series of such experiments in glycylglycine media in which preparation of the tissue occupied 14 min, the first slices had inulin spaces, in μl/100 mg slice, of 52·5 ± 2·7 (*b*); the second, 58·2 ± 3·6 (*b*) and the third, 57 ± 3·4. Their increase in weight during incubation showed similar differences. No consistent differences were found in non-inulin space or in oxygen uptake. Fig. 2 shows individual instances of the difference in swelling in the first and second slices.

Added substances. Observations made incidentally to other experiments suggested that protamine and gangliosides affected tissue fluids (THOMSON and MCILWAIN, 1961). Results under the present conditions (Table 2) show that protamine markedly

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diminished swelling by an action almost entirely confined to the inulin space. Gangliosides increased the inulin space, and this could be prevented by protamine.

Comparison with other methods of handling the tissue. In the previous study of electrically stimulated tissue (CUMMINS and MCILWAIN, 1961), experimental conditions differed from those of Table 2 in two ways. Slices were cut with a blade lubricated with the incubating medium and floated in the medium prior to draining and weighing at the beginning of the experiment; and the experiments were terminated by floating the slices in cold isotonic sucrose to remove adhering medium prior to analysis for Na and K.

TABLE 2.—EFFECT OF PROTAMINE AND GANGLIOSIDES ON TISSUE FLUIDS

Slices were prepared (time occupied: 14 min) as in Table 1, in glycylglycine-buffered glucose salines with the additions specified. Incubation, analysis, and expression of results: as in Table 1, except for *P* values: these are not stated when they are greater than 0·05.

Measurement (per unit fresh wt)	Additions (mg/ml) to media				*P* values
	(*a*) None	(*b*) Protamine, 0·15	(*c*) Ganglioside, 0·3	(*d*) Protamine, 0·15 Ganglioside, 0·3	
Respiration (μmoles O_2/g/hr)	89 ± 8 (18)	86 ± 6 (12)	94 ± 12 (12)	86 ± 8 (12)	—
Increase in wt (mg/100 mg)	39 ± 4 (18)	29 ± 5 (12)	41 ± 3·5 (12)	32 ± 3 (12)	$a - d$, <0·001; $c - d$, <0·001
Inulin (μl occupied/100 mg)	56 ± 3 (15)	48 ± 4 (9)	62 ± 7 (12)	50 ± 5 (12)	$a - d$, <0·001; $c - d$, <0·001
Volume not occupied by inulin (μl/100 mg)	62 ± 4 (15)	59 ± 3 (9)	59 ± 5 (12)	62 ± 4 (12)	$b - d$, <0·05 $c - d$, <0·05

Experiment A of Table 3 examines the first process. The slicing and floating in fluid was timed and this case involved 13 min in contact with fluid at room temperature prior to weighing (this is much longer than is usual). This period permits an additional 25 per cent of fluid uptake (Fig. 3). If this uptake is the only relevant process, values for respiration on the basis of the floated-weight of the tissue may be converted to those on a fresh weight basis by multiplying by 1·25; this has been done in column (*b*), Expt. A, Table 3, giving values which are in agreement with those of direct observation, quoted in column (*c*). The increase in weight in column (*b*) is derived by (*a*) × 1·25 + 25, and agrees approximately with column (*c*). The inulin spaces, again derived by multiplying (*a*) by the factor 1·25, also agree; the relationship between tissues prepared by the two methods thus appears relatively straightforward.

In experiment B of Table 3 the second process is examined, though release was to 0·32M-glucose rather than to sucrose, to minimize interference in the inulin assay. The glucose rinse is seen to remove inulin corresponding in amount to 11 μl of fluid/100 mg original weight of tissue. Rather greater quantities of Na and Cl are removed; potassium is unaffected, as would be expected from its intracellular location.

Tissue incubated and electrically stimulated

The experiments of Table 4 were carried out with quick-transfer electrodes and with minimal exposure of tissues to fluid before their incubation at 37°. About 2 min only were required for floating and mounting the tissue in the apparatus in oxygenated medium at 37°. Consequently the tissue, even after incubation, had

TABLE 3.—METHODS OF PREPARING AND RINSING CEREBRAL SLICES

Experiments A were carried out in glycylglycine-buffered media with tissue prepared in the same length of time by the two methods described in the experimental section; for calculation of column (c), see text. Experiments B were in bicarbonate media with tissue prepared without added fluid; slices (a) were drained at the end of the experiment and slices (b) were released into isotonic glucose at this stage, before analysis.

Measurement (per unit fresh wt except in column A(a))	A. Tissue drained for analysis			B. Cut dry	
	(a) Sliced in fluid; referred to wet wt.	(b) Calculated from (a) to dry-cut tissue	(c) Dry cut	(a) Slices drained	(b) Released into glucose
Respiration (μmoles O_2/g hr)	68 ± 6 (15)	85 ± 8 (15)	86·3 ± 10 (6)	—	—
Increase in wt (mg/100 mg)	29·5 ± 6 (15)	62 ± 8 (15)	57·7 ± 4 (6)	29·2 ± 6 (27)	—
Inulin (μl occupied/100 mg)	50·8 ± 4 (15)	63·5 ± 5 (15)	59·3 ± 3 (6)	53·7 ± 6·4 (27)	42·1 ± 12 (20)
Volume not occupied by inulin (μl/100 mg)	60 ± 4 (15)	78·3 ± 5 (15)	78·3 ± 6 (6)	53·7 ± 4·8 (27)	66·6 ± 9·1 (20)
Na (μequiv/g)	—	—	—	150 ± 7·8 (16)	129 ± 15·5 (6)
K (μequiv/g)	—	—	—	74·5 ± 6 (19)	74·5 ± 3·8 (6)
Cl (μequiv/g)	—	—	—	107 ± 10 (27)	81 ± 6·7 (14)

swollen relatively little (Table 4). The composition of the control tissue after incubation is compared (Table 4) with those of tissues which had received electrical pulses at the end of their incubation, and with others which had received pulses but had been allowed a period for recovery. The groups did not differ significantly in their swelling, their inulin space, or in the volume not occupied by inulin.

By contrast, the Na and K of the tissue changed markedly: 14 μequiv of K was lost and 12 μequiv of Na gained/g tissue as a result of applying pulses. During incubation after pulses ceased, some 60 per cent of the K lost was recovered, and about 60 per cent of the Na was extruded.

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DISCUSSION

Tissue fluids

Additional fluid becomes associated with the slice, and evidence has been given above that it is of the following categories.

(1) *Adhering medium.* Direct weighing indicated that the slice increased in weight by 8·5 per cent after brief immersion and draining. Slices which had been incubated in inulin-containing media and rinsed briefly in cold isotonic glucose had a quantity of

TABLE 4.—EFFECT OF ELECTRICAL STIMULATION ON TISSUE FLUIDS, Na, K AND Cl OF INCUBATED GUINEA PIG CEREBRAL CORTEX

Slices were cut dry, weighed, and successively placed in bicarbonate-glucose saline, mounted in quick-transfer electrodes, and oxygenated at 37°. After 40 min incubation pulses were applied to slices (b) and (c) for 10 min. Slices (b) were then released, drained, weighed and placed in trichloroacetic acid. At this time (40 min incubation) pulses to the electrodes carrying slices (c) were switched off and 5 min later they, and also slices (a), were released and treated as slices (b). Mean values are followed by S.D.; 4 determinations each.

Measurement (per unit fresh wt)	Group of slices		
	(a) Control	(b) Stimulated	(c) Stimulated with subsequent recovery period
Increase in wt (mg/100mg)	28·1 ± 3·5	25·9 ± 6·5	29·4 ± 4·4
Inulin (μl occupied/100 mg)	50·6 ± 7·2	50·6 ± 4·7	52·4 ± 7·2
Volume not occupied by inulin (μl/100 mg)	57·5 ± 3·7	55·3 ± 5·7	57 ± 3
Na (μequiv/g)	144 ± 12	156 ± 8	149 ± 7
K (μequiv/g)	71·5 ± 5·2	57·5 ± 2·4	65 ± 3·4
Cl (μequiv/g)	100 ± 11·4	103 ± 10·3	107 ± 7·7

inulin, corresponding to 11 μl/100 mg tissue, removed. These values do not differ significantly and the slices as handled in the present experiments are taken in subsequent calculations as carrying 10 μl of adhering medium/100mg of fresh tissue. (It should be noted that it is readily possible to drain the tissue more completely, but that this has not been done in the present experiments as a longer time is required).

(2) *Swelling during preparation.* This occurred while the tissue was in contact with medium and air (not O_2) at room temperature, and began at the rate of 2·5 μl/100mg/min (Figs. 1 and 3). Subsequently the rate of increase fell, but the swelling was not reversed on incubation under good metabolic conditions with oxygen and glucose. When, after incubation in inulin-containing media, the nature of the change during preparation could be appraised (Fig. 4) the swelling was found to be in the non-inulin space. This probably implies intracellular changes and emphasizes the value of speedy preparation of the tissue.

(3) *Swelling on incubation.* The changes described under (2) are halted on restoring good metabolic conditions by incubation at 37° in oxygenated glucose media. A slow swelling then proceeded, at 0·1 μl/100 mg tissue/min, that is, at only 4 per cent of the initial rate of swelling during preparation. This was distinct from (2) not only in the speed and circumstances of its occurrence, but also because it was parallelled by increase in the inulin-space of the tissue (Fig. 3).

Interpretation; change with applied agents. Thus a slice of 100 mg weighed without contact with fluid, prepared in 5 min and subsequently incubated for 60 min would have increased in weight in category (1) by 10 mg; in (2) by 12·5 mg; and in (3) by 6 mg, and a weight of 128·5 mg would be anticipated after incubation. Values of 126–130 mg were observed (Tables 3 and 4). In such tissue the inulin space was observed as 51 μl/100 mg but of this, 16 μl were known to be due to changes (1) and (3). Presumably the remaining 35 μl/100 mg represents the fraction of the original tissue fluid which is accessible to inulin, and 45 μl/100 mg is thus non-accessible to inulin. After preparation and incubation, this has been increased to 57·5 μl by change (2).

Following the interpretation quoted at the beginning of this paper, the space occupied by inulin may be taken as extracellular and the majority of that not occupied as intracellular. It is then understandable that under the poor metabolic conditions in which changes (2) occur, when the energy-rich substances of the tissue are known to be depleted, the fluid changes should be intracellular. Intracellular swelling was observed also by PAPPIUS and ELLIOTT (1956) in the absence of oxygen and in the presence of glutamate; glutamate also causes loss of the tissue's phosphocreatine (MCILWAIN, 1951; WOODMAN and MCILWAIN, 1961). The protamine and ganglioside preparations on the contrary, had greatest effect on extracellular fluids. They have already been interpreted as acting at the cell membrane (WOLFE and MCILWAIN, 1961; THOMSON and MCILWAIN, 1961) and are of large molecular weight in aqueous solution, a factor likely to limit their access to the cell interior.

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Electrolytes

It is instructive to express the Na, K and Cl measured in the tissue in the experiments of Tables 3 and 4, in terms of the volumes of tissue fluid computed to be occupied and not occupied by inulin. This has been done calculating first (Table 5, (i)) the quantities of Na and Cl which would be in the inulin-space on the supposition that this had the same composition as the incubating medium. The Na and Cl in the non-inulin space can then be obtained by subtraction from the analyses of Tables 3 and 4, as in (ii), Table 5. With the values of Table 4 which exhibit the effects of electrical excitation and recovery, this calculation has been made for the individual slices and the data of Table 5 (ii) are given with the S.D. derived from these individual values. These S.D. are much smaller than those for the total Na and Cl of Table 4, indicating a major cause of variation to be in the inulin space, and presumably in the adhering medium. The Na values of Table 5 (ii) give clear demonstration of Na entry to the non-inulin space with excitation, and its extrusion during a subsequent period without excitation. The Cl values did not change significantly.

Concentration in non-inulin space. As the volume of the tissue compartment not occupied by inulin has been computed above, the quantities of electrolytes just calculated can be expressed as concentration (Table 5, (iii)). Further, the concentration ratios between this compartment and the incubating medium can be derived (Table 5, iv).

These ratios emphasize the high concentration gradient maintained in K; substitution in the Nernst equation

$$E = \frac{RT}{F} \ln \frac{[K_0]}{[K_i]}$$

gives the potential E corresponding to the ratio of external to internal potassium salts, $[K_0]/[K_i]$, as −81 mv. Calculation from values computed from *in vivo* observations (ECCLES, 1957; K_0, 5·5 and K_i, 150 mM) gives E as −87 mv. Both these are within the overlapping range of potentials observed by intracellular micropipette electrodes *in vivo* and *in vitro* (LI and MCILWAIN, 1957; HILLMAN and MCILWAIN, 1961).

TABLE 5.—ELECTROLYTE CONCENTRATIONS IN INULIN AND NON-INULIN SPACES

Values computed from results in Tables 3 and 4. In these experiments, the medium after incubation was found to contain: Na, 165; K, 6·6; and Cl, 134 μequiv/ml. Values (ii) from Table 4 are calculated individually for each of the 12 slices concerned and mean values are followed by S.D.

Measurement	Value in slices			
	Normal		Electrically stimulated (Table 4)	
	Table 3B(a)	Table 4	No recovery	Recovery
(i) Na of inulin-space (μequiv/g)	89	84	84	86·5
Cl of inulin-space (μequiv/g)	72	67	67	70
(ii) Total Na less that of (i) (μequiv/g)	61	60 ± 3·3	72 ± 2·1	62 ± 4·3
Total Cl less that of (i) (μequiv/g)	35	33 ± 3·8	36 ± 4·8	37 ± 4·2
(iii) Na in non-inulin space (mM)	113	104	130	107
K in non-inulin space (mM)	138	125	103	115
Cl in non-inulin space (mM)	65	58	65	65
(iv) Ratios (Na, non-inulin)/(Na, medium)	0·69	0·63	0·79	0·65
(K, non-inulin)/(K, medium)	20·9	18·9	15·6	18·0
(Cl, non-inulin)/(Cl, medium)	0·49	0·43	0·49	0·49
(v) Computed chloride space (μl/100 mg)	80	74	77	80
Na in supposed non-chloride space (mM)	62	65	94	59
K in supposed non-chloride space (mM)	258	212	197	224

Sodium salts, by contrast, are not distributed according to the Nernst equation. The ratio diverges in the same sense as in tissues *in vivo*, and presumably for the same reason: that osmotic work is performed in extruding Na^+. The completeness of the extrusion, if the non-inulin space is taken as intracellular, is however much less than *in vivo* when ratios of 0·096 have been calculated (WOODBURY, 1955); direct observation in the squid axon gives a ratio $[Na_i]/[Na_0]$ of 0·11 (HODGKIN, 1958). The concentration ratio now observed in chloride ion also diverges from that which probably obtains *in vivo*. Tissue spaces *in vivo* are often calculated on the basis of an assumption that chloride is entirely extracellular. If, indeed, the ion was distributed passively according to the Nernst equation the ratio $[Cl_i]/[Cl_0]$ would be about 0·05. A ratio of 0·073 is reported for the squid axon (HODGKIN, 1958).

Computation on the basis of a non-chloride space. Values (Table 5, (v)) calculated on the assumption just referred to lead to the conclusion that Cl^- occupies about 26 μl/100 mg fresh tissue beyond the space occupied by inulin. This leaves a non-Cl space of only 30 μl/100 mg, which appears too small to be the total intracellular volume. These estimates indicate the difficulty encountered in regarding the tissue as comprising only intracellular spaces from which specific 'markers' are excluded and extracellular spaces to which they have full access. In a whole organ, as for example the diaphragm (CREASE and NORTHOVER, 1961) the fluid of extracellular spaces does not necessarily have the same content of the 'markers' as does an external fluid.

Hyaluronic acid in dilute aqueous solution has been observed by OGSTON and PHELPS (1961) to make a proportion of solvent unavailable to inulin, and comparable factors may operate in the extracellular spaces of neural tissues. If, however, 30μl/100mg tissue was the total intracellular space, giving K_i the high though still feasible values indicated, Na_i would remain high and would give a ratio $[Na_i]/[Na_0]$ which remained much above those computed for neural tissue *in vivo*.

The relatively sluggish movements of inulin into and out of the tissue (Figs. 2 and 3) suggest that changes in extracellular fluids occurring during experimental periods of less than 30 min would be only partly reflected by changes in inulin content. This may be the case during the experiments of Table 4 when electrical pulses were applied for 10 min then 5 min was allowed for recovery. In appraising the changes in tissue Na, it is therefore important to note that during these experiments neither tissue weight nor chloride changed significantly. (During longer electrical stimulation, under different conditions, THOMSON and MCILWAIN, 1961, found an increase in tissue fluid.) Also, when computed on the basis of non-chloride space, tissue sodium again increased on stimulation and diminished during recovery.

It may be concluded that, although the tissue *in vitro* reproduces well the K concentration and the Na and K movements on excitation and recovery which are important parts of its behaviour *in situ*, relations between the Na and Cl of the unexcited tissue and its environment have not yet been reproduced satisfactorily *in vitro*. There are already indications that, *in vivo*, permeability to Na is open to modification by constituents of normal blood and by added drugs (WOODBURY, KOCH and VERNADAKIS, 1958). Comparable phenomena have been described in other tissues, especially in muscle (see for example, CREESE and NORTHOVER, 1961). In neural tissues the Na differential is important to excitability and transmission, and its further study in the isolated sections of cerebral cortex is in progress.

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SUMMARY

1. Metabolic experiments with slices of guinea pig cerebral cortex *in vitro* involve association of additional fluid with the tissue by (i) adhering medium (ii) swelling during preparation of the tissue; and (iii) swelling during incubation.

2. Media containing inulin were used to characterize the fluid changes; inulin itself needed 20 min or more to equilibrate with the tissue. The fluid absorbed during preparation was in a space not accessible to inulin, while the fluid absorbed during incubation was accessible to inulin.

3. In incubation media containing protamine, tissue swelling was less than in normal media, while in media containing ganglioside preparations, swelling was

greater; both these changes were in fluid accessible to inulin. Application of electrical stimulation for 10 min had little effect on fluids but caused loss of K and gain of Na by the tissue.

4. The chloride of the tissue did not appear to be confined to the space accessible to inulin; it and the Na were concluded to exist intracellularly to a greater extent than in the brain *in vivo*.

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THE DISTRIBUTION OF PHOSPHORUS-CONTAINING LIPID COMPOUNDS IN THE HUMAN BRAIN

S. BALAKRISHNAN,* H. GOODWIN and J. N. CUMINGS
Department of Chemical Pathology, Institute of Neurology, The National Hospital, Queen Square, London, W.C.1

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MANY investigations have been carried out on the lipid composition of the brain in different species of animals, to determine the changes which occur during myelination and early development and in different lipid storage diseases (BRANTE, 1949; CUMINGS, 1955 1960*b*; EDGAR, 1956; FOLCH, 1955; JOHNSON, MCNABB and ROSSITER, 1949). The different methods employed by the various workers for extraction, fractionation and determination of the individual lipid components present difficulties in correlating the data obtained by them.

The recent paper chromatographic method developed by DAWSON (1960) allows lipid fractions to be separated and quantitatively estimated on the basis of the phosphorus content. The one-dimensional technique of DAWSON was used in the present study of brain material from humans of various ages and from humans afflicted by certain neurological disorders.

MATERIALS AND METHODS

All analyses were made on the white matter of fresh human brains obtained at autopsy except in a few experiments where comparison was made with formalin-fixed material. Lipids were extracted with chloroform–methanol (2:1, v/v) and water soluble lipids were removed by the procedure of FOLCH, LEES and SLOANE STANLEY (1957). The hydrolytic and chromatographic procedures were carried out exactly as described by DAWSON (1960) but only ascending chromatography was used. Water saturated phenol–acetic acid–ethanol (100:10:12, by vol) was used as solvent for the mild alkali labile and mild acid labile phospholipids and water saturated phenol was used for the mild alkali and acid stable phospholipids. The detection of the phosphorus-containing spots and estimations of phosphorus in them was also carried out as described by DAWSON (1960). Calculations and correction factors suggested by him were used for all fractions except phosphatidylserine which has not been corrected for the phosphatidic acid and polyglycerol phospholipid (which usually travel together with phosphatidylserine as a single spot). Each estimation was made in duplicate and the average result is given. Throughout there has been an attempt to follow closely the terminology used by DAWSON and to use as far as possible the same general pattern in recording results so that comparison can be readily made between the results for man and those recorded for animals.

RESULTS

Normal human brain

The lipid phosphorus distributions in the white matter of the normal brain at various ages are presented in Table 1 and Fig. 1. The term plasmalogen as used here, includes choline, ethanolamine and serine plasmalogen which were listed separately by DAWSON. The figures given are related to 100 g of dry tissue and not to a percentage of total lipid phosphorus.

* Present address: Department of Biochemistry, All-India Institute of Mental Health, Bangalore 2, India.

Total lipid phosphorus. In this small series there is a steady increase with age up to 6 years though the increase from birth to 1 year was most marked. After 6 years the total lipid phosphorus appears to remain relatively constant.

Phosphatidylcholine (lecithin). A fairly pronounced decrease in the lecithin phosphorus occurs during the first 8 months after birth then the level remains fairly constant.

Phosphatidylethanolamine. A diminution in this fraction of phospholipid (i.e., the di-acyl-ester form) occurs during the first year and then the level tends to rise slightly.

Phosphoinositide. The level of this phospholipid fraction is found to be constant throughout the series studied.

TABLE 1.—PHOSPHOLIPID DISTRIBUTION IN WHITE MATTER OF HUMAN BRAIN
(Results expressed as mg lipid phosphorus/100 g dry tissue)

	New-born	3 months	8 months	1 year	6 years	14 years	Adult
Total lipid phosphorus	720	750	770	900	1020	1000	990
Water (%)	89	84	78	77	71	70	70
Phosphatidylcholine	317 (44)	285 (38)	224 (29)	252 (28)	224 (22)	210 (21)	198 (20)
Phosphatidylethanolamine	138 (19)	120 (16)	69 (9)	83 (9)	112 (11)	80 (8)	100 (10)
Phosphatidylserine	115 (16)	105 (14)	138 (18)	143 (16)	160 (16)	170 (17)	149 (15)
Phosphoinositide	29 (4)	30 (4)	24 (3)	29 (3)	30 (3)	30 (3)	29 (3)
Plasmalogens	50 (7)	90 (12)	122 (16)	184 (20)	143 (13)	130 (13)	134 (13)
Sphingomyelin	22 (3)	45 (6)	100 (13)	120 (13)	152 (15)	200 (20)	178 (18)
Unidentified mild alkali and acid stable phospholipids	22 (3)	23 (3)	62 (8)	54 (6)	82 (8)	120 (12)	79 (8)
Recovery (% of total lipid phosphorus analysed)	96	93	96	95	89	95	88

Values in parentheses represent percentage of total lipid phosphorus

Phosphatidylserine. A small increase occurs between 3 and 8 months and subsequently there is little change in levels.

Plasmalogens. A very definite increase takes place up to the age of one year followed by a slight decrease to the adult level. Ethanolamine and choline plasmalogens were also estimated separately and it was found that almost 90 per cent of the total plasmalogens were of the former type.

Sphingomyelin. From a very low level at birth there is a sharp increase up to 8 months which is followed by a more gradual increase up to 14 years.

Niemann-Pick's disease

Similar data were obtained for the brain and spleen of a case of Niemann-Pick's disease and this is compared (Table 2) with the distribution in a normal case of very similar age. In cerebral white matter the total lipid phosphorus of the Niemann-Pick case was about 15 per cent lower than normal. Among the constituent phospholipids, the proportions of phosphatidylethanolamine and plasmalogens in the Niemann-Pick white matter were slightly lower than in the normal case. The proportions of the other fractions including sphingomyelin were more or less the same in both cases.

The total lipid phosphorus in the Niemann-Pick cortex is also below the normal. The proportionate distribution of the different fractions is slightly different from that of white matter. The lecithin phosphorus and phosphatidylserine phosphorus are

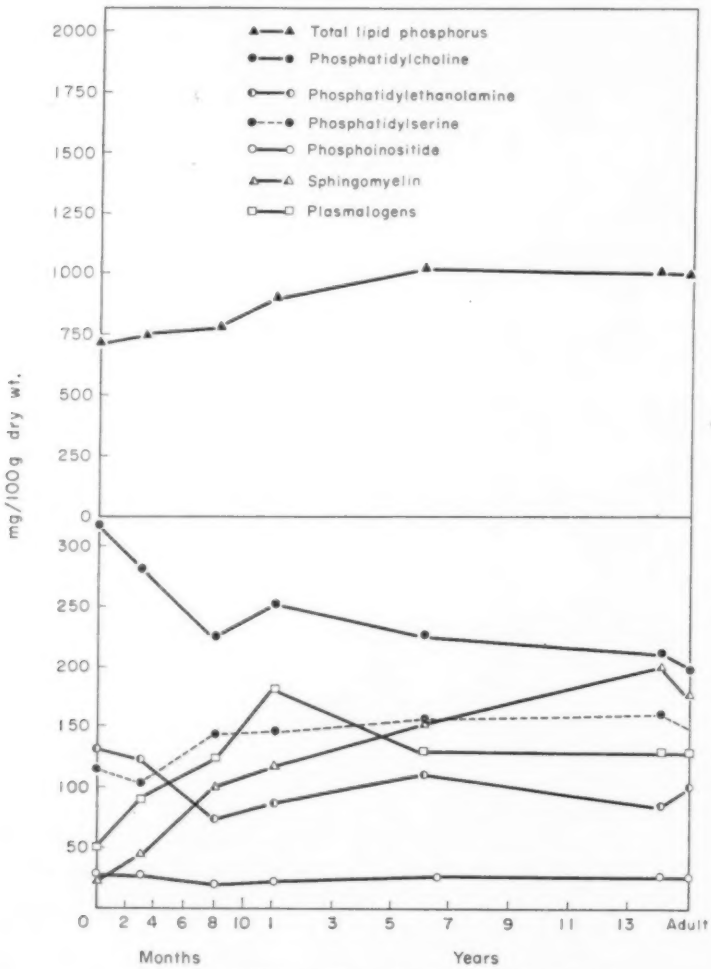

FIG. 1.—Lipid phosphorus distribution in human brain.

slightly lower in percentage of the total in the Niemann-Pick case, and sphingomyelin is slightly higher, in comparison with the normal.

The Niemann-Pick spleen contains more total lipid phosphorus than the normal spleen. The predominant fraction of the phospholipid in the Niemann-Pick spleen is, however, sphingomyelin which constitutes 45 per cent of the total lipid phosphorus as compared with 15 per cent in the normal spleen. The actual sphingomyelin phosphorus value for the Niemann-Pick spleen is nearly 6 times the normal value.

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TABLE 2.—PHOSPHOLIPID DISTRIBUTION IN ORGANS OF A CASE OF NIEMANN-PICK'S DISEASE COMPARED WITH A NORMAL CASE
(Results expressed as lipid phosphorus in mg/100 g dry weight)

	Niemann-Pick (8 years)			Normal (6 years)		
	Brain		Spleen	Brain		Spleen
	Cerebral white matter	Cortex		Cerebral white matter	Cortex	
Total lipid phosphorus	850	632	570	1000	865	309
Water (%)	71	81	73	70	83	78
Phosphatidylcholine	204 (24)	202 (32)	93 (16)	220 (22)	302 (36)	99 (32)
Phosphatidylethanolamine	68 (8)	95 (15)	63 (11)	110 (11)	130 (14)	37 (12)
Phosphatidylserine	136 (16)	57 (9)	34 (6)	160 (16)	95 (11)	40 (13)
Phosphoinositide	26 (3)	Traces	Traces	30 (3)	Traces	Traces
Plasmalogens	94 (11)	88 (14)	29 (5)	140 (14)	104 (12)	41 (13)
Sphingomyelin	136 (16)	102 (16)	262 (45)	150 (15)	112 (13)	46 (15)
Unidentified mild alkali and acid stable phospholipids	94 (11)	57 (9)	29 (5)	80 (8)	51 (6)	16 (5)
Recovery (% of total lipid phosphorus analysed)	89	95	88	89	92	90

Values in parentheses represent percentage of total lipid phosphorus

TABLE 3.—PHOSPHOLIPID DISTRIBUTION IN THE BRAIN IN A CASE OF SUBACUTE ENCEPHALITIS AND A CASE OF AMAUROTIC FAMILY IDIOCY
(Results expressed as lipid phosphorus in mg/100 g dry white matter)

	Subacute encephalitis (8 years)	Amaurotic family idiocy (8 years)
Total lipid phosphorus	668	1000
Water (%)	79	76
Phosphatidylcholine	174	250
Phosphatidylethanolamine	74	100
Phosphatidylserine	120	170
Phosphoinositide	20	30
Plasmalogens	53	100
Sphingomyelin	94	150
Unidentified mild alkali and acid stable phospholipids	87	110
Recovery (% of total lipid phosphorus analysed)	92	91

Subacute encephalitis and amaurotic family idiocy

In the white matter of the brain from a case of subacute encephalitis (Table 3), the levels of total phosphorus and all the phosphorus-containing compounds are lower than normal, while in the case of amaurotic family idiocy no significant changes in distribution are observed.

Formalin fixed material

In view of the difficulties in always obtaining fresh brain tissue, it was considered useful to compare the lipid phosphorus distribution in fresh and in formalin-fixed white matter in brains from 1-year old infants. The formalin was not neutralized and was faintly acid. Fixation was for a period of 5 days. Data for both cases (Table 4) show that the total lipid phosphorus is about 10-12 per cent lower in the fixed material. Among the individual fractions, the values for phosphatidylserine and ethanolamine plasmalogen in fixed material are noticeably lower. The other components do not seem to be affected.

TABLE 4.—DISTRIBUTION OF LIPID PHOSPHORUS IN FRESH AND FIXED WHITE MATTER OF HUMAN BRAIN

(Results expressed as lipid phosphorus in mg/100 g dry weight)

	Case 1 Age 1 year		Case 2 Age 1 year	
	Fresh	Fixed	Fresh	Fixed
Total lipid phosphorus	790	709	780	698
Water (%)	69	72	70	73
Phosphatidylcholine	229	234	202	222
Phosphatidylethanolamine	63	71	70	63
Phosphatidylserine	150	63	124	63
Phosphoinositide	32	31	23	21
Plasmalogens	126	78	118	56
Sphingomyelin	111	116	117	119
Unidentified mild alkali and acid stable phospholipids	55	71	78	95
Recovery (% of total lipid phosphorus analysed)	97	93	94	92

DISCUSSION

Data relating to myelination are important not only in relation to such disease processes as the lipidoses and some of the demyelinating conditions but also because they add to the present scanty knowledge on this subject. It has been said from histological studies that myelination commences around the fourth month of intra-uterine life (KUNTZ, 1950) and stops at the end of the second year of life (VON MONAKOW, 1905). Yet in a recent review (NATHAN and SMITH, 1955) it was shown that while these facts may be true for the cortico-spinal tract and the median longitudinal bundle there are at least 8 other tracts for which no definite knowledge concerning the date of myelination is available. The process of myelination in man has been discussed by LUMSDEN (1957) from a histological aspect but there is little reliable evidence which demonstrates at what time the various myelin lipids are incorporated into the myelin sheath. It is known moreover that in animals, such as the rabbit, the histological and biochemical evidences of myelination do not run parallel to each other (EDGAR, 1956, 1957).

It is therefore of the utmost importance that detailed biochemical data should be collected and the observations on the increase in total lipid phosphorus, as determined by the procedure used here, during the early stages of development are thus of some

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importance. These data are in general agreement with those of earlier workers (JOHNSON, MCNABB and ROSSITER, 1949; BRANTE, 1949; CUMINGS, 1960*b*; CUMINGS, GOODWIN, WOODWARD and CURZON, 1958).

JOHNSON *et al.* (1949) and BRANTE (1949) reported that the amount of lecithin in the white matter of the brain was less in the adult than in the newborn. The findings reported here over a wider range of age groups confirm and expand the earlier observations.

With regard to the cephalin group of phospholipids (phosphatidylserine, phosphatidylethanolamine and phosphoinositide), when expressed on the basis of dry weight,

TABLE 5.—CEPHALIN AND SPHINGOMYELIN DISTRIBUTION IN THE WHITE MATTER OF HUMAN BRAIN AT DIFFERENT AGES

(Results expressed as g/100 g fresh tissue)

Age	Phosphatidyl-ethanolamine	Phosphatidyl-serine	Phospho-inositide	Plasma-logens	Sphingomyelin
Newborn	0·38	0·32	0·08	0·14	0·06
3 months	0·48	0·42	0·12	0·36	0·18
8 months	0·38	0·76	0·13	0·67	0·54
1 year	0·48	0·85	0·17	1·06	0·70
6 years	0·81	1·16	0·22	1·04	1·10
14 years	0·60	1·28	0·23	0·98	1·50
Adult	0·74	1·11	0·22	1·01	1·30

(All phosphorus values multiplied by a factor of 25 for conversion into phospholipid)

the group as a whole shows no great increase with increase in age. The plasmalogen phosphorus, however, is found to increase significantly. BRANTE (1949) reported an increase in level of cephalins (cephalin 'A') from the foetal to the adult stage; it is possible that in his method of analysis plasmalogens were included. This would account for the increase in total 'cephalin' concentration in the adult brain observed by him.

Some comment concerning the plasmalogen fraction is necessary. Although the figures of the results are recorded in the same manner as DAWSON's (1960) it is recognised from more recent work (PIETRUSZKO and GRAY, 1960; DAVENPORT and DAWSON, 1961) that some of the total plasmalogen phosphorus may be present in part of the 'sphingomyelin' phosphorus and also in the 'unidentified' fraction. If this is so then suitable corrections would have to be made; for instance, the plasmalogens in the newborn (see Table 1) would rise from 50 to 83 and those in the adult from 134 to 302 in the white matter. If similar corrections are made for the other ages the values obtained here would agree more closely with those presented by other workers (WEBSTER, 1960). It is also pertinent to mention here that there was no evidence of any significant amount of glycerylphosphorylserine in the chromatogram of the plasmalogen fraction.

The cephalins, plasmalogens and sphingomyelin as taken from Table 1 (phosphorus values multiplied by 25) are expressed (Table 5) on the basis of the fresh weight of tissue. The 3–4 fold increase in the sum total of cephalins and plasmalogens with increase in age compares well with the 6-fold increase reported by BRANTE (1949). However, the increase appears to be predominantly due to the plasmalogen fraction

and to a lesser extent to the phosphatidylserine fraction. The predominance of ethanolamine plasmalogen over the choline plasmalogen again agrees well with the recent observations of WEBSTER (1960).

The striking increase in sphingomyelin phosphorus with age is in agreement with earlier observations (BRANTE, 1949; CUMINGS, GOODWIN, WOODWARD and CURZON, 1958), although the absolute values tend to be lower than by other methods. This difference is probably accounted for by the appearance of 'unidentified' mild alkali and acid stable phospholipids appearing along with the sphingomyelin fraction. BRANTE (1949) reported considerable quantities of an alkali resistant phospholipid fraction in brain which he designated as 'cephalin B'. ANSELL and SPANNER (1961) have also shown that sphingomyelin forms only part of the fraction yielding water-soluble phosphorus after vigorous acid hydrolysis of the phospholipids stable to mild alkali and acid hydrolysis. SCHMIDT *et al.* (1959) described a procedure for the quantitative partition of tissue phospholipids and discussed the discrepancies encountered in the sphingomyelin determinations by their earlier method of hydrolysis (SCHMIDT, BENOTTI, HERSHMANN and THANNHAUSER, 1946). They suggest that incomplete hydrolysis of the plasmalogens could be responsible for the higher sphingomyelin values obtained in the earlier investigations ('cephalin B').

The observations with regard to lipid distribution in the organs in Niemann-Pick's disease deserve some comment. Definite sphingomyelin increases in brain and spleen have been reported by KLENK (1934;1939–40). CUMINGS (1957) reported sphingomyelin increase only in the cortex of a case of Niemann-Pick's disease with a fairly normal distribution in white matter. CROCKER (1961) in his recent investigations on Tay-Sachs' and Niemann-Pick's diseases reported increase of sphingomyelin in the cortex only in the classical type of infant patients; in the other 3 forms of the disease only very mild abnormalities occurred in grey matter; increases of sphingomyelin in liver and spleen were found to be general in almost all forms of this disease. More investigations in cases of Niemann-Pick's disease at various ages and at varying stages of development of the disease need to be carried out using DAWSON's method of analysis.

The result recorded here for the child of 8 years would be in agreement with the findings of CROCKER if the portion of 'sphingomyelin' due to the admixture of plasmalogens, as already commented upon, were subtracted: the levels of sphingomyelin in the cortex of both the normal subject and the diseased patient are then almost identical. In view of the loss of total lipid phosphorus, however, the proportion of sphingomyelin relative to total lipid phosphorus in the patient (8·1%) is higher than in the control subject (6·4%).

A comparison between the histological findings in Niemann-Pick's disease with the biochemical results described here is of some interest. BIELSCHOWSKY (1928) found the nerve changes in this disease similar to those in Tay-Sachs' disease, namely a distension of nerve cells with a lipid substance. This has been confirmed by others using modern histochemical techniques. The case described here showed storage cells in the cerebral cortex in which PAS positive material was present. Other workers (NORMAN, 1958; DIEZEL, 1960) have described similar lesions and there can be little doubt that this stored lipid is ganglioside. DIEZEL in one of his cases showed that copper phthalocyanin (CP) positive material was present in the brain and that this CP material was sphingomyelin, which would be in agreement with the relative increase of sphingomyelin in the brain of the case reported in this paper.

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The histological changes seen in Tay-Sachs' disease occur mainly in the cerebral cortex, the white matter showing little alteration in the early stages (DIEZEL, 1960). These facts are reflected in the lack of biochemical abnormality found in the case mentioned here.

The loss of phosphorus associated with phosphatidylserine and plasmalogens in fixed material appears to support the statement of BRANTE (1949) with regard to loss of cephalins 'possibly phosphatidylserine' during fixation in formalin. This loss of plasmalogens may be due to hydrolysis in an acid medium and if so this would tend to increase the apparent content of phosphatidylethanolamine and the true content of phosphatidylethanolamine would accordingly be lower than the recorded figure. The other constituent phospholipids do not seem to be affected in brains fixed in formalin for 5 days or less. CUMINGS (1960*a*) reported a similar loss in the total phospholipid in formalin-fixed brains, with sphingomyelin and lecithin levels being relatively unaffected, indicating thereby a preferential loss of the cephalin fraction. He also showed that it was possible to recover the lost phospholipids from the fixing medium.

SUMMARY

The paper chromatographic technique of DAWSON (1960) for the separation and quantitative estimation of phosphorus has been applied to the study of lipid phosphorus distribution in the white matter of the human brain.

In a series of 7 cases ranging in age from the newborn to adult, the phospholipid fractions were analysed and striking increases with increasing age were observed in the proportion of plasmalogen and sphingomyelin phosphorus. Lecithin phosphorus on the other hand was found to diminish with increase in age. These results are discussed in relation to the findings of other workers.

Analyses of lipid phosphorus in brain have also been made in cases of Niemann-Pick's disease, subacute encephalitis, and amaurotic family idiocy and also in formalin fixed brains.

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FURTHER STUDIES OF THE PROPERTIES OF THE ENCEPHALITOGENIC COMPONENT(S) OF BOVINE NERVOUS TISSUE*

M. B. LEES, L. AMADUCCI† and B. H. WAKSMAN‡
McLean Hospital Research Laboratory, Belmont, Massachusetts, the Neurology Service, Massachusetts General Hospital, and the Departments of Neuropathology and Bacteriology and Immunology, Harvard Medical School, Boston, Massachusetts

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EXPERIMENTAL allergic encephalomyelitis (EAE) is a well defined disease entity which can be produced by the injection of central nervous system tissue into a variety of animals under specific conditions. Encephalitogenic activity has been shown to be associated with central white matter but is absent from grey matter and from unmyelinated tissue. However, the elucidation of the chemical nature of the encephalitogenic agent has been a difficult problem and the results of several groups of investigators apparently conflict. On the one hand, the existence of a chloroform–methanol soluble antigen active in mice, guinea pigs and rabbits has been reported by TAL and OLITSKY (1952), GOLDSTEIN *et al.* (1953), and WAKSMAN *et al.* (1954) respectively. The evidence suggested that the chloroform–methanol soluble antigen might be proteolipid in nature; but, at the same time, it was concluded that, if proteolipids had antigenic activity, it was only a small, specific fraction of the proteolipids which was active (WAKSMAN *et al.*, 1954; FOLCH and LEES, 1959). By the use of a suitable bio-assay method, the material extracted from bovine white matter by chloroform–methanol was shown to have less antigenic activity than the fresh tissue when injected into a rabbit, and was still less active in the guinea pig. In both species, it produced a dose-response curve with a slope which was different from that of fresh tissue (WAKSMAN *et al.*, 1954). These observations suggested that there had been a qualitative change in the antigen during the extraction procedure or, alternatively, that the encephalitogenic activity of the fresh tissue was due to more than one antigen, only one antigen being extracted by chloroform–methanol. On the other hand, it was shown later that an antigen active in guinea pigs could be isolated from the residue remaining after extraction of the tissue with a series of lipid solvents (KIES, ALVORD and ROBOZ, 1958*a*, *b*). In addition, ROBOZ, HENDERSON and KIES (1958) prepared a water-soluble, lipid free, glycoprotein which was active in guinea pigs but inactive in rabbits. Recently, LIPTON and STEIGMAN (1959) reported the isolation from petroleum ether extracts of bovine spinal cord of a nitrogen-rich fraction which was extremely active in guinea pigs. The present experiments have been carried out in order to test the encephalitogenic activity in rabbits of fractions similar to those extracted by LIPTON, to study further the nature and behaviour of the

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† Visiting Scholar under the Fulbright Act 1958–1960. Present address: Clinica Neuropsichiatrica, Florence, Italy.

‡ National Neurological Research Foundation Scientist.

petroleum ether soluble antigen, and to compare its properties with those of the chloroform–methanol soluble antigen in both rabbits and guinea pigs.

MATERIALS AND METHODS

Analytical methods. Phosphorus, galactose and cholesterol were estimated respectively by modifications of the methods of SPERRY (1942), SØRENSON and HAUGAARD (1933), and KINGSLEY and SCHAFFERT (1949). The amount of protein was computed from the amount of α-amino acid nitrogen released on acid hydrolysis. The difference between the α-amino acid nitrogen before and after hydrolysis as determined by the ninhydrin procedure of VAN SLYKE *et al.* (1941) was multiplied by the empirical factor 9 to convert it to a figure which represents the amount of protein in the sample. Protein values reported previously (WAKSMAN *et al.*, 1954; FOLCH and LEES, 1959) were obtained using the conversion factor 12, so that, if comparisons are to be made with the earlier data, the figures must be adjusted accordingly. This change in the numerical value of the factor is the result of studies carried out on proteolipid proteins in the course of other work (FOLCH, 1959).

Preparation of chloroform–methanol extracts of nervous tissue. Washed chloroform–methanol tissue extracts were prepared as described previously (FOLCH, LEES and SLOANE STANLEY, 1956). In brief, fresh or fresh-frozen bovine brain white matter (medulla oblongata and/or subcortical white matter) or spinal cord were stripped of meninges and homogenized with 19 vol of chloroform–methanol (2:1, v/v) in a Waring blendor for 3 min at room temperature. The extract was filtered, and to the clear filtrate was added one-fifth its volume of water. After mixing and centrifugation, two clear phases were obtained. The upper phase consisted mainly of water and methanol, and contained all the non-lipid contaminants and most of the strandin from the original extract; the lower phase consisted mainly of chloroform, and contained all the tissue lipids and proteolipids. When this chloroform phase was dried by vacuum distillation of its solvents, the proteolipids remained intact and could be redissolved in chloroform–methanol. Bovine or human sciatic nerves were extracted at room temperature as described above with the exception that, before extraction, the nerve was minced, frozen to the temperature of dry ice, and ground in a mortar maintained at the same temperature.

Preparation of petroleum ether extracts of nervous tissue. Fresh or fresh-frozen tissue samples were homogenized for 3 min in an explosion-proof Waring blendor with 5 vol of cold petroleum ether (analytical reagent, b.p. 30–60°, repurified as described by LEES *et al.*, 1959). The homogenate was stirred for 1 hr in a container surrounded by ice, transferred to tubes, and centrifuged at 3000 rev/min (1700 g) at 4° for one hour. The cloudy supernatant (petroleum ether extract at 3000 rev/min (1700 g) was decanted, dried by distillation of its solvents *in vacuo*, and tested for antigenic activity. The petroleum ether-insoluble tissue residue was tested directly for antigenic activity by combining the residue still wet with petroleum ether and tissue water with the adjuvants (*vide infra*). In other experiments, the petroleum ether-insoluble tissue residue was extracted with sufficient chloroform–methanol to form a single liquid phase. After filtration, water was added to make the total amount of water (including the tissue water) a quarter of the volume of the chloroform–methanol. After mixing and centrifugation, two phases separated; the upper phase was discarded and the solvents from the lower phase were removed by vacuum distillation.

Fractionation of petroleum ether tissue homogenate. A separation of the antigenic activity into several fractions was brought about by means of high speed centrifugation of the petroleum ether homogenate. To this effect, a petroleum ether tissue homogenate prepared as described above was transferred to lusteroid tubes and centrifuged at 20,000 rev/min (40,000 g) for 1 hr at approximately 4° in a Spinco Model L centrifuge (head No. 21). Three fractions were thus obtained: (1) a clear supernatant, (2) a well-packed residue, and (3) a gel-like layer between the residue and the clear supernatant. The supernatant, together with the gel, was decanted and the residue washed three times with petroleum ether. After each of the washings, the gel continued to appear; the supernatant and gel were decanted together and combined with the original supernatant and gel. (In some experiments, the washings were tested separately.) The washed residue was dried *in vacuo* over $CaCl_2$. The combined supernatant plus gel fractions were re-centrifuged at 20,000 rev/min (40,000 g) the resulting supernatant, which, in some preparations, showed a slight trace of opalescence, was drawn off with a pipette and its solvent removed by evaporation. The gel, which remained at the bottom of the tube, was dried *in vacuo* over $CaCl_2$.

Alternatively, in some cases, the petroleum ether tissue homogenate was first centrifuged at 3,000 rev/min (1700 g) and the cloudy supernatant thus obtained was re-centrifuged at 20,000 rev/min,

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(40,000) Under these conditions, after the high speed centrifugation a gel and a supernatant were obtained, with only barely detectable amounts of residue. Thus, the cloudy supernatant at 3,000 rev/min appears to be comparable to the gel plus the supernatant and these are separable from one another by high speed centrifugation.

Preparation and injection of antigenic material. Fresh tissue or a tissue fraction was ground in a mortar with mineral oil (Bayol F, a light, technical grade mineral oil, obtained from the Standard Oil Company of New Jersey) containing heat-killed bacilli (Jamaica 22 strain,* 3 mg/ml) to give a homogeneous suspension or emulsion, which could be diluted with mineral oil containing bacilli to any desired concentration of antigen. The concentration of a given fraction was always expressed in terms of an equivalent weight of fresh tissue. A stock solution of antigen was prepared by grinding 2 g of fresh tissue, or its equivalent, with 3 ml of oil and bacilli. The volume of injected material was kept constant, different dose concentrations being achieved by appropriate dilution of the stock solution with oil and tubercle bacilli. Groups of rabbits, usually five, were injected with approximately 0·3 ml of antigenic mixture intradermally, divided among the four toe-pads. This portion contained the equivalent of 200 mg of fresh tissue and was taken as the standard dose level since it produced the maximum disease response in rabbits. Groups of Hartley guinea pigs were injected intradermally at a single site over the sternum with 0·1 ml of the stock solution which had been diluted 10-fold. This portion was equivalent to 6·67 mg of fresh tissue, the standard dose level for these animals. The differences in inoculation technique precluded any attempt to compare the amounts of material required to produce disease in the two species.

In some experiments, fractions were incorporated into two additional types of adjuvant mixtures comparable to those employed by KIES *et al.* (1957) and LIPTON (1953). These mixtures contained the following constituents (by vol):

	KIES adjuvant	LIPTON adjuvant
Mineral oil†	8·0	8·5
Emulsifier	4·0‡	1·5§
Water	8·0	10·0
Tubercle bacilli	3 mg/ml (final concentration)	

† Bayol F: a light, technical grade mineral oil, obtained from the Standard Oil Company of New Jersey.

‡ Arlacel A: mannide mono-oleate, obtained from the Atlas Powder Company, Wilmington, Delaware.

§ Aquaphor: a mixture of cholesterol and cholesterol esters in aliphatic hydrocarbons, obtained from Duke Laboratories, South Norwalk, Connecticut.

In each instance, the bacilli were first suspended in oil, the emulsifier was added to the oil (Aquaphor was first liquefied by warming), and the aqueous phase was then added in small portions with continuous mixing by drawing in and out of a syringe. Before the final preparation of the emulsion, the test fraction was incorporated into either the oil or aqueous phase, in order to compare the encephalitogenic activity of mixtures prepared by different procedures; these, in turn, were compared with the encephalitogenic activity of fractions prepared for injection by our standard technique.

Evaluation of immunologic response. Rabbits or guinea pigs were observed daily following their inoculation with an antigenic mixture, and were killed within 3–4 days after the appearance of neurological symptoms. Survivors were killed approximately 30 days after injection. In rabbits, a cisternal puncture was performed at the time of autopsy and the total protein and cell count in the cerebrospinal fluid was determined by standard methods (KOLMER and BOERNER, 1945). From each animal, tissue sections were taken at three levels of the spinal cord, with the accompanying spinal roots and sensory ganglia, stained with haematoxylin and eosin, and studied for histological changes. Sections of sciatic nerve were examined from animals inoculated with peripheral nerve fractions. The observed lesions were classified as + to +++ on the basis of their number and intensity. Each animal was then assigned a graded score, from 0 to 6, based on the day of onset of neurological

* Obtained through the courtesy of Dr. Jules Freund.

symptoms and the severity of the disease as estimated histologically (WAKSMAN *et al.*, 1954). A score of 1 was assigned when there were questionable histological lesions, *ante mortem* signs of disease without concomitant histological findings, or cerebrospinal fluid abnormalities, i.e., more than 50 mg% total protein and/or 15 cells/mm, in the absence of other changes. Cerebrospinal fluid abnormalities were disregarded in rabbits which proved to have lesions of *Encephalitozoon cuniculi*.

Although there were differences in the severity of the disease produced by the fractions tested in the present study, all the fractions produced disease which was clinically and histologically indistinguishable from that produced by fresh tissue. The character of the lesions and their distribution remained remarkably constant, and one could not distinguish between disease produced by a decreased dose of an active material and that produced by a large dose of a relatively inactive antigen.

TABLE 1.—ENCEPHALITOGENIC ACTIVITY OF VARIOUS BOVINE TISSUE FRACTIONS INJECTED INTO RABBITS AND GUINEA PIGS

Fraction tested*	Average graded score	
	Rabbits	Guinea pigs
A. White matter		
(1) fresh tissue	5·5	4·6
(2) washed $CHCl_3$:CH_3OH tissue extractives	2–4	1·5–1·8
(3) petroleum ether tissue extractives (3000 rev/min)	5·2–5·4	5·2
(4) petroleum ether-insoluble tissue residue	2·8	3·0
(5) $CHCl_3$:CH_3OH extractives of A–(4)	1·5	0
B. Spinal cord		
(1) fresh tissue	4·0	4·6
(2) washed $CHCl_3$:CH_3OH tissue extractives	4·3	0·8
(3) petroleum ether tissue extractives (3000 rev/min)	4·8–5·4	4·0–5·8
(4) petroleum ether-insoluble tissue residue	4·4	4·8
(5) $CHCl_3$:CH_3OH extractives of B–(4)	0	0

* All fractions were injected at a standard dose level which for rabbits corresponded to 200 mg of fresh tissue and for guinea pigs to 6·7 mg of fresh tissue.

RESULTS

Antigenic potency of petroleum ether extracts at 3,000 rev/min (1700 **g**). The antigenic potency of the solutes from petroleum ether extracts (3,000 rev/min, 1700 **g**) of bovine brain white matter or spinal cord is essentially the same as that of comparable doses of fresh tissue when injected into either rabbits or guinea pigs. Not only are the graded scores which are obtained after injection of the two types of material similar, but the dose-response curves are almost superimposable (Table 1 and Fig. 1). On the other hand, the solutes from washed chloroform–methanol tissue extracts do not produce the same degree of disease in guinea pigs as they do in rabbits. The disease which is produced in rabbits is slightly milder and of later onset than that produced by the fresh tissue, whereas in guinea pigs, the response to the chloroform–methanol soluble antigen is minimal (Table 1 and Fig. 1). In both species, the decrease in activity

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appears to be related to a qualitative change in the antigen since the graded score is not increased by injecting larger doses of the extract (WAKSMAN *et al.*, 1954).

Although the petroleum ether tissue extractives are as active in producing EAE as is the fresh tissue, nevertheless, the petroleum ether insoluble tissue residue is still antigenic. Even after extraction of this residue with fresh portions of petroleum ether until essentially no additional solutes can be removed, the petroleum ether insoluble tissue residue remains antigenic in both rabbits and guinea pigs, although at a reduced level. When this residue is extracted with chloroform–methanol and the solutes are tested for antigenic activity, they are relatively inactive in guinea pigs but active in rabbits, the

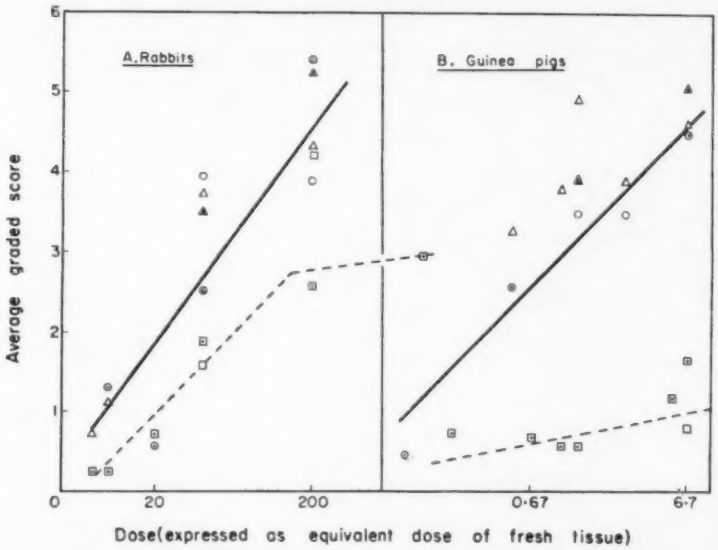

FIG. 1.—Titration, in rabbits and guinea pigs, of encephalitogenic activity of fractions obtained from bovine white matter and spinal cord.

○ = fresh tissue; brain white matter.
○ = fresh tissue; spinal cord.
△ = petroleum ether extractives (3,000 rev/min) from brain white matter.
△ = petroleum ether extractives (3,000 rev/min) from spinal cord.
⊡ = chloroform–methanol extractives from brain white matter.
□ = chloroform–methanol extractives from spinal cord.

activity in the latter being less than that of fresh tissue. Thus, the average graded score and the species specificity of the chloroform–methanol extractives from the petroleum ether insoluble tissue residue are similar to those of the chloroform–methanol extractives of fresh tissue (Table 1).

Chemical properties of petroleum ether tissue extracts (3,000 rev/min., 1700 g) The results of the analyses on the solutes from both petroleum ether and chloroform–methanol tissue extracts of bovine brain white matter and spinal cord are shown in Table 2. It can be seen that the total tissue lipids are not present in the petroleum ether extracts of either white matter or spinal cord; i.e., only 50–60 per cent of the total lipids of brain white matter are extracted by petroleum ether, while 80 per cent of the total lipids of spinal cord are extracted; the remaining lipids are extractable from the petroleum ether-insoluble tissue residue with chloroform–methanol.

At the time this study was initiated, an explosion-proof Waring blendor was not available to us and it was felt that, because of the high flammability of petroleum ether, a conventional Waring blendor would be dangerous to use. Therefore, in the early experiments the tissue was homogenized with petroleum ether in a Lourdes homogenizer with a quart size Mason jar. In these experiments, it was found that homogenization for successive 3 min periods resulted in the extraction of the following amounts of solutes into the petroleum ether extract at 3,000 rev/min (1700 **g**) (results expressed as

TABLE 2.—CHEMICAL COMPOSITION AND ANTIGENIC ACTIVITY OF SOLUTES FROM VARIOUS BOVINE BRAIN WHITE MATTER AND SPINAL CORD EXTRACTS

	$CHCl_3$:CH_3OH tissue extractives*	Petroleum ether tissue extractives	$CHCl_3$:CH_3OH extractives of petroleum ether-insoluble tissue residue*
Brain white matter			
As % of fresh tissue			
total solutes	20·0	11·5	6·8
phosphatides	9·0	4·6	2·9
proteolipid protein	1·5	0·78	0·3
As % of total solutes			
phosphatides	45	40	42
proteolipid protein	7·5	6·8	4·5
Graded score in rabbits	2–4	5·2–5·4	1·5
Graded score in guinea pigs	1·5–1·8	5·2	0
Spinal cord			
As % of fresh tissue			
total solutes	29·6	24·2	5·4
phosphatides	13·0	11·5	2·7
proteolipid protein	1·2	1·7	0·27
As % of total solutes			
phosphatides	44	47	50
proteolipid protein	4·0	6·8	5·1
Graded score in rabbits	4·3	3·0–5·4	1·8
Graded score in guinea pigs	0·8	4·0–5·8	0

* These extracts were washed with water as described above (FOLCH *et al.*, 1956) before being analysed.

per cent of the total lipids): spinal cord, 66·3, 14·0, and 1·4; brain, 33·8, 7·5, 6·0, and, after homogenization for an additional 40 min, 4·1. It thus appears more difficult to extract brain white matter with petroleum ether than it is to extract spinal cord.

Stability of petroleum ether tissue extracts (3,000 rev/min, 1700 **g**). The encephalitogenic activity of the petroleum ether extracts of brain and spinal cord appears to be relatively stable, both to the action of $CHCl_3$:CH_2OH and to storage for relatively long periods of time. After removal of the solvents from the petroleum ether tissue extract *in vacuo*, the resulting residue can be redissolved in chloroform–methanol or chloroform–methanol–water mixtures, dried under conditions whereby proteolipids remain intact (FOLCH *et al*, 1951), and tested for antigenic activity. The solutes thus treated still retain the antigenic potency of the original petroleum ether extract both in rabbits and in guinea pigs, i.e., mere treatment of the solutes from the petroleum ether extract with chloroform–methanol–water mixtures does not appear to result in any loss of

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TABLE 3.—ENCEPHALITOGENIC ACTIVITY OF PETROLEUM ETHER TISSUE EXTRACTS INJECTED INTO RABBITS AND GUINEA PIGS*

Fraction	Graded score	
	Rabbits	Guinea pigs
Brain		
(1) Petroleum ether-soluble tissue extractives	5·2	5·2
(2) Fraction (1) redissolved in $CHCl_3$:CH_3OH (2:1) and dried	5·4	3·9
Spinal Cord		
(3) Petroleum ether-soluble tissue extractives	5·4	4·0
(4) Fraction (3) redissolved in $CHCl_3$:CH_3OH (2:1) and dried	4·3	4·4
(5) Fraction (3) redissolved in $CHCl_3$:CH_3OH:H_2O (8:4:3) lower phase dried	3·6	5·0

* Each fraction was tested on at least five animals and, in this series of experiments, every animal tested showed disease.

TABLE 4.—EFFECT OF STORAGE FOR 6 MONTHS AT ROOM TEMPERATURE ON ENCEPHALITOGENIC ACTIVITY OF A BOVINE SPINAL CORD PETROLEUM ETHER EXTRACT

Fraction tested	Equivalent dose of fresh tissue (mg)	EAE in guinea pigs	
		No. with EAE / No. tested	Average graded score
Fresh tissue	1·0	—	about 3·0*
Petroleum ether tissue extract prepared 7/7/58 and tested immediately thereafter	about 1·0	5/5	3·6
Same extract tested 1/12/59	about 1·0	5/5	4·2
'Skin' from above extract collected and tested 1/12/59	6·7	3/5	1·8

* Estimated from data at other dose levels (see Fig. 1)

their encephalitogenic activity (Table 3). On the other hand, extraction of either the fresh tissue or the petroleum ether-insoluble tissue residue with chloroform–methanol results in a decrease in activity. Furthermore, the solutes from the petroleum ether tissue extract remain antigenically active after storage of the extract at room temperature for at least six months (Table 4).

Despite this apparent stability of the antigenic activity, physical changes become visible in the extract within a few days of its preparation. A white material begins to appear as filmy strands which are suspended throughout the solution. As more of the material appears, it forms heavier strands which may remain suspended or, after long periods of time, may tend to settle to the bottom of the cylinder. The formation of this material, which for convenience has been designated as 'skin', can be delayed

considerably by storage of the extracts at 4°, in which case no change in the appearance of the extract is evident for several months. The 'skin' cannot be redissolved in either chloroform–methanol–water mixtures or petroleum ether, and has relatively low antigenic activity compared to that of the original extract. Various preparations contain between 2·5% and 4·5% nitrogen, and between 0·76% and 1·04% phosphorous.

Antigenic potency and chemical properties of fractions obtained after high speed centrifugation of petroleum ether homogenates. Numerous unsuccessful attempts were made to concentrate the antigen(s) present in the white matter petroleum ether extracts (3,000 rev/min, 1700 **g**) by means of fractionation with various solvents. In every case, the antigenic activity either disappeared completely or was found at decreased levels in all fractions and, concomitantly, the fractions exhibited changes in their solubility properties. For example, after removal of acetone-soluble material from the petroleum ether extractives, a procedure which might be expected to remove only cholesterol, the acetone-insoluble residue was no longer completely soluble in petroleum ether. Neither of the two fractions thus obtained was as active as the original petroleum ether extract, but the portion of the solutes which had become insoluble in petroleum ether was the more active of the two fractions when tested in either rabbits or guinea pigs. Simple emulsification of the petroleum ether tissue extractives in water also resulted in an appreciable loss of activity, a fact which had been observed previously for the $CHCl_3$:CH_3OH extractives (Waksman, 1957). It was felt, therefore, that it might be more satisfactory to attempt a separation by centrifugation rather than by solvent fractionation. If the antigenic activity was related to the presence of proteolipid, the high specific gravity of the protein moiety of the proteolipid as compared with that of free lipid should make it possible to concentrate the antigenic activity by high speed centrifugation.

The three fractions (supernatant, gel and residue) obtained by centrifugation of the petroleum ether homogenate at 20,000 rev/min (40,000 **g**) showed uniform activity when injected into either rabbits or guinea pigs at the standard dose level. However, titration of each of the fractions indicated that in the rabbit, at lower dose levels, the gel was the most active fraction: at one-fifth the standard dose level, the gel was still as active as the standard dose of fresh tissue while the other fractions showed a marked decrease in activity (Table 5). In the guinea pig, both the gel and the supernatant showed high activity at one-fifth the standard level. When the gel and supernatant mixtures were centrifuged at still higher speeds (36,000 rev/min, 85,000 **g**) the bulk of the solids were found in the gel, the yield of solids in the supernatant amounting to only 8 mg per gram of fresh tissue; under these conditions, the supernatant was antigenically inactive even when injected at high dose levels. If the results are evaluated on the basis of the amount of solids injected into the animal rather than of equivalent doses of fresh tissue, it is still clear that, in the rabbit, the fraction obtained as a gel after centrifugation of the petroleum ether extract at 20,000 rev/min (40,000 **g**) shows more antigenic activity than do any of the other fractions. Although only 2·1 mg of solids was injected into each rabbit, a maximal antigenic response was obtained (graded score = 4·2). In the guinea pigs, both the gel fraction and the fraction soluble upon centrifugation at 20,000 rev/min (40,000 **g**) show high activity at one-fifth the standard dose. In these cases, maximal response is obtained with as little as 0·05–0·07 mg of solids.

Only a limited number of chemical analyses have been carried out on the fractions obtained by centrifugation at 20,000 rev/min (40,000 **g**) but it is evident that each of the

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fractions consists of a mixture of lipids with more proteolipid in the gel than in the supernatant (Table 5).

Use of different adjuvants. Since the role played by the adjuvant mixture in producing an encephalitogenic response has never been fully elucidated, and since various investigators have prepared their adjuvant mixtures in different ways, it appeared essential to compare the antigenic potency of tissue fractions combined with the adjuvant mixture by our standard procedure with these same fractions prepared for injection by other procedures. To this effect, the solutes from both petroleum ether and

TABLE 5.—CHEMICAL COMPOSITION AND ANTIGENIC POTENCY OF FRACTIONS OBTAINED BY HIGH SPEED CENTRIFUGATION OF PETROLEUM ETHER HOMOGENATES OF BRAIN WHITE MATTER

Fraction tested	Yield mg/g fresh tissue	Chemical composition as % of solids in fraction	Average graded score			
			Rabbits		Guinea pigs	
			Dose 1*	Dose 0·2*	Dose 1*	Dose 0·2*
Fresh tissue			5·5	3·3†	4·7	3·3†
20,000 rev/min						
Petroleum ether extractives	38	33% phosphatide 49% cholesterol 3% proteolipid protein	3·0	0·5	4·3	4·5
Gel	52	42% phosphatide 18% cerebroside 19% cholesterol 10% proteolipid protein	3·6	4·2	3·8	3·8
Washings from gel	28	44% tissue proteins and 56% lipids and proteolipids	3·6	1·1	2·0	1·0
Petroleum ether-insoluble tissue residue	118		3·8	1·8	3·9	2·3
36,000 rev/min						
Petroleum ether extractives	8		0·7‡		1·7‡	
Gel	24		3·5		4·1	

* Dose 1 refers to injection of the fraction at a standard dose level which in the case of rabbits corresponds to 200 mg of fresh tissue and in the case of guinea pigs to 6·7 mg of fresh tissue; dose 0·2 refers to injection of the fraction at one-fifth the standard dose level.

† Estimated from data at other dose levels.

‡ Injected at 3 times standard level.

chloroform–methanol tissue extracts were injected into animals at different dose levels, using our standard procedure for the preparation of the adjuvant mixture, and, for comparison, using water-in-oil adjuvant mixtures in which the test fraction was incorporated into either the aqueous or the oil phase. It was found that when the petroleum ether-soluble material was injected into rabbits, a slightly greater encephalitogenic response was sometimes obtained when the fractions had been combined with the water-in-oil adjuvant mixture than with our standard mixture (Fig. 2). However, in guinea pigs, a markedly decreased encephalitogenic response was obtained when the petroleum ether-soluble material had been combined with the water-in-oil adjuvant mixtures as

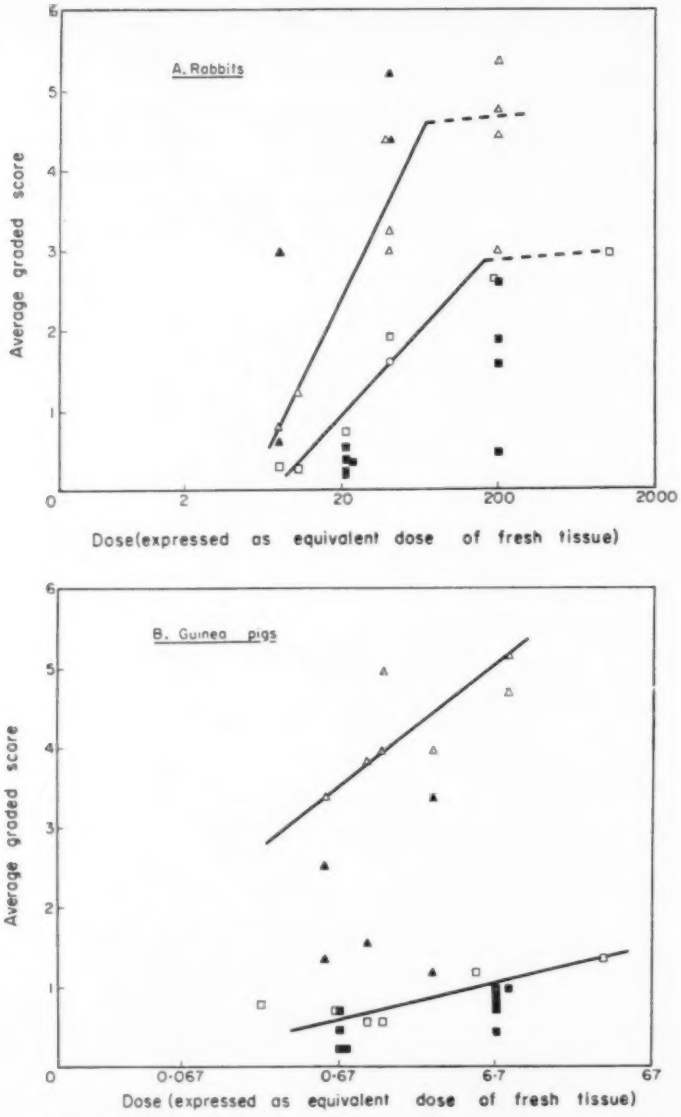

FIG. 2.—Effect of different adjuvants on the antigenic potency of bovine brain white matter tissue extracts (see text).
△ = petroleum ether tissue extract prepared for injection by our standard procedure.
▲ = petroleum ether tissue extract prepared for injection by other procedures.
□ = chloroform–methanol tissue extract prepared for injection by our standard procedure.
■ = chloroform–methanol tissue extract prepared for injection by other procedures.

compared with our own procedure. In both species, when chloroform–methanol tissue extractives were injected, the greatest encephalitogenic response occurred when our standard procedure was used. These observations were clearer in the case of rabbits than in guinea pigs, since the latter gave a relatively low encephalitogenic response to chloroform–methanol tissue extractives under any conditions. The

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lowest scores were obtained when the fractions had been emulsified in the aqueous phase rather than in the oil phase during the preparation of the mixture, a finding which is consistent with previous observations that emulsification of various fractions in the course of their purification resulted in a loss of encephalitogenic activity (WAKSMAN, 1957).

Antigenic potency of sciatic nerve extracts (Table 6). Although it is known that injections of fresh sciatic nerve result in the appearance of peripheral neuritis (EAN) in both rabbits and guinea pigs (WAKSMAN and ADAMS, 1956), repeated attempts to produce a similar condition in animals injected with the solutes from chloroform–methanol extracts of bovine sciatic nerve have been unsuccessful. Since these extracts

TABLE 6.—PRODUCTION OF EXPERIMENTAL ALLERGIC NEURITIS (EAN) IN RABBITS BY BOVINE AND HUMAN SCIATIC NERVE FRACTIONS

	Equivalent dose of fresh tissue	No. with EAN / No. tested	Average graded score
Bovine sciatic nerve			
Fresh tissue	200	3/3	5·0
	40	5/5	5·6
	8	3/4	3·0
Washed $CHCl_3$:CH_3OH extractives	200	0/10	0
Petroleum ether extractives	200	2/5	1·2
	40	0/5	0
Human sciatic nerve			
Fresh tissue	200	4/4	3·3
Petroleum ether extractives	200	6/6*	3·0

* The antigen was autoclaved before its injection into half of this group of rabbits.

contain only negligible amounts of proteolipid (0·6 mg proteolipid protein per gram fresh tissue, FOLCH, LEES and CARR, 1958), their inability to produce neuritis would be consistent with the hypothesis that the antigenic agent for EAN is also proteolipid in nature. Petroleum ether extracts of bovine sciatic nerve were found to contain somewhat larger amounts of protein (5 mg proteolipid protein per gram fresh tissue, AMADUCCI, unpublished), and injection of the solutes from these extracts has resulted in the production of definite allergic neuritis in rabbits but not in guinea pigs. Although, at comparable dose levels, the neuritis in the rabbit is milder than that produced by fresh bovine nerve, it is the first time that experimental allergic neuritis has been produced by any type of tissue extract rather than by the fresh tissue itself.* Similar experiments using the solutes from a petroleum ether extract of human sciatic nerve showed them to be more active than the bovine material since they resulted in the production of disease in all the rabbits tested and gave a higher average graded score.

DISCUSSION

The name *proteolipid* has been given to a group of lipoproteins which are soluble in chloroform–methanol and insoluble in water, and which consist chemically of a lipid

* BOGDANOVE and CLARK (1960) reported the occurrence of central nervous system lesions in a small number of guinea pigs injected with fractions obtained from the chloroform–methanol extract of horse peripheral nerve.

moiety and a protein moiety. Other known lipoproteins are soluble in water or salt solutions and insoluble in, or destroyed by, organic solvents, and thus exhibit solubilities akin to those of protein. In contrast, the solubility properties of the proteolipids are similar to those of lipids. Proteolipids are widely distributed tissue components occurring in highest concentration in cerebral white matter. Their concentration in cortical grey matter is about one-fifth that of cerebral white matter, while peripheral nerve contains, at most, only one-twentieth to one-thirtieth the proteolipid concentration of cerebral white matter (Folch *et al.*, 1958). In view of the behaviour of proteolipids during various fractionation procedures, it has been postulated that they consist of a protein core surrounded by layers of lipid associated with the protein through different kinds of bonds. Information is available on the chemical composition of the total proteolipids from brain white matter, but relatively little is known about the protein-lipid linkages or about the existence of different types of proteolipids. This lack of knowledge is related, in part, to the difficulties encountered in handling proteolipids; whereas under certain conditions they are resistant to the action of strong chemicals, in other instances they readily become insoluble. For example, they can withstand limited treatment with 6 N-HCl, but are split by simple drying from $CHCl_3$:CH_3OH:H_2O solutions in which a two-phase system is formed during the process of evaporation of the solvents. In an analogous manner, the antigenic activity can withstand autoclaving, but often disappears or decreases after mild solvent fractionation procedures, such as extraction with acetone or ether.

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According to the definition of Folch and Lees (1951), the petroleum ether tissue extract described in this paper contains proteolipid: it contains protein which had been extracted with an organic solvent, and after removal of that solvent, the residue is soluble in chloroform–methanol. However, the nature of the protein and of the protein-lipid linkages in chloroform–methanol and in petroleum ether tissue extracts has not been established, and therefore, no conclusion can be drawn as to the relationship between the proteolipids extracted by the two different solvents. The significant factor in the appearance of antigenicity may be related to the orientation of the protein and the lipid moieties which constitute proteolipids, or to the actual chemical composition of the proteolipids, or to a combination of the two, and all these factors must be taken into consideration in evaluating the changes in antigenicity observed in the course of fractionation procedures.

The data reported in this paper demonstrate that, after extraction of the tissue with petroleum ether, antigenic activity can be obtained in three fractions, namely, the petroleum ether-soluble fraction, the petroleum ether-insoluble tissue residue, and a fraction of intermediate solubility, the 'gel'. The proportion of total solids and antigenic activity in each fraction has been shown to vary with the speed of centrifugation. Since the antigenic activity of the petroleum ether-soluble fraction and the gel is essentially the same as that of fresh tissue, a reasonable hypothesis might be that petroleum ether acts by breaking down an active tissue component into smaller fragments which vary in size but maintain essentially the structural relationships characteristic of fresh tissue and necessary for antigenicity. Chloroform–methanol, on the other hand, may rupture bonds at some other point resulting in a loss of, or a decrease in, certain configurations required for antigenicity. Support for this idea can be found when one considers the difference in the behaviour of the two solvent systems towards water: whereas petroleum ether and water are completely immiscible with one another,

$CHCl_3$:CH_3OH (2:1) takes up approximately 8% water. Thus, $CHCl_3$:CH_3OH removes water from the tissue, and, in doing so, may alter physico-chemical relationships that are necessary for antigenicity. In contrast, petroleum ether, being immiscible with water, could either extract the solutes, leaving the tissue water as a separate fraction or, alternately could extract a lipid-protein complex still containing 'bound' water. That the latter occurs, is suggested by the appearance of two phases upon the addition of methanol to the petroleum ether extract (unpublished data). Whereas, petroleum ether and methanol are completely miscible, the addition of small amounts of water to the methanol results in the formation of a two-phase system. Thus, it appears that the petroleum ether tissue extract contains water in a form which is miscible with petroleum ether but which can be removed by methanol. These observations may be related in some way to the factors necessary for preserving the structural integrity of the antigen.

SUMMARY

The solutes from the petroleum ether extract (3,000 rev/min, 1700 **g**) of bovine white matter or spinal cord are as active in producing EAE in rabbits and guinea pigs as is a corresponding dose of fresh tissue; in both species the dose-response curves obtained after injection of the petroleum ether extractives are comparable to those obtained with fresh tissue.

High speed centrifugation of petroleum ether tissue homogenates results in the appearance of three fractions: (1) a supernatant, (2) a gel, and (3) the residue. Although all fractions show antigenic activity, the gel appears to be the most encephalitogenic in rabbits.

Treatment of the petroleum ether extract with chloroform–methanol–water mixtures does not appear to result in the loss of encephalitogenic activity which is associated with the extraction of either the fresh tissue or the petroleum ether-insoluble tissue residue with chloroform–methanol.

The solutes from the petroleum ether extract of bovine or human sciatic nerve produce experimental allergic neuritis in rabbits but not in guinea pigs.

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INTRALAMINAR DISTRIBUTION OF ADENOSINETRIPHOSPHATASE ACTIVITY IN HUMAN FRONTAL ISOCORTEX*

HELEN H. HESS and ALFRED POPE
The McLean Hospital Research Laboratory, Belmont, Mass. and the Department of Neuropathology, Harvard Medical School, Boston, Massachusetts

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PREVIOUS publications from this laboratory have described the progress of experimental work designed to outline the biochemical architecture of mammalian cerebral cortex. To this end, the intracortical distributions of certain enzymes important in brain metabolism are being studied in human anterior frontal cortex and in rat somatosensory isocortex by the methods of quantitative histochemistry. A primary objective is to correlate the biochemical architecture with the cyto- and myeloarchitecture and with the electrophysiological properties of the cortex. The work serves a wider purpose of building up a body of information helpful in assessing possible metabolic or structural alterations of human cortex in certain nervous and mental diseases.

Adenosinetriphosphatases, which release inorganic phosphate from ATP† in the absence of other added substrates, were among the enzymes chosen for study because early work with myosin suggested involvement of ATPases in the specialized functions of highly differentiated tissues. A survey of ATPase activity in rat somaesthetic cortex was conducted using two assay systems, the first having Ca^{++}, the second Mg^{++} as activating ion (HESS and POPE, 1959). It was found that Ca-ATPase showed maximal activity in layer I, lesser elevations of activity in layers III(a), III(b), V(c) and VI(a), and an overall decline of activity as a function of subpial depth. This pattern is consistent with occurrence of Ca-ATPase activity in finely subdivided cortical plexuses of axons and dendrites. Mg-ATPase activity, on the other hand, was greatest in layers II, III, and IV, subsidiary peaks being seen in layers V(b) or V(c) and in VI(a), a pattern consistent with localization chiefly in neuronal perikarya and dendrites, and at axon terminals.

This paper reports the details of an analogous study of Ca-ATPase and Mg-ATPase activities in human cortical biopsy specimens excised during frontal lobotomy. The distributions of the two enzyme activities in human cortex bear a striking resemblance to their respective distributions in rat cortex, and presumably may be interpreted similarly as indicating that in human brain Ca^{++} and Mg^{++} predominantly activate separate enzymes having different cytological locations and functional roles.

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† The abbreviations used are: ATP, adenosinetriphosphate; AChE, acetylcholinesterase; NAD nicotinamide adenine dinucleotide (formerly called diphosphopyridine nucleotide, DIXON, 1960). The terms Ca-ATPase and Mg-ATPase refer to enzymes which hydrolyse ATP in the presence of Ca^{++} and Mg^{++} respectively. As determined in tissue slices, these activities are to be considered as indices of the rates of ATP-utilizing reactions under the conditions of assay.

MATERIAL AND METHODS

The cortical specimens were excised during the course of prefrontal lobotomies upon patients having various types of mental illness. They were representative of homotypical, eugranular frontal cortex from the area designated 9 by BRODMANN, FDm by VON ECONOMO and KOSKINAS (VON BONIN, 1950), and IEfs by BAILEY and VON BONIN (1951). The procedures for removing the biopsy specimens and handling them prior to sampling were described previously (POPE, CAVENESS and LIVINGSTON, 1952). As soon as the refrigerated specimen was received at the laboratory, a block of cortex was selected for enzyme study and frozen in a dry ice box. The remaining tissue was fixed and used for neuropathological study. The methods for sampling and sectioning the cortex were those used previously (POPE *et al.*, 1952; POPE, 1959; HESS and POPE, 1960). The block of frozen cortex was transferred to a cold room at $-12°$. A cylinder, 2 mm in diameter and oriented with its long axis at right angles to the pial surface, was cut from the cortical block by means of a metal punch. After being mounted in a rotary microtome, the cylinder was sectioned into slices 40 μ thick, passing from layer I through the successive layers of cortex and into white matter. The 40 μ thick frozen sections were studied in consecutive sets of four: the first slice of each set was stained with buffered thionine; the second was assayed for Ca-ATPase; the third was desiccated and weighed on a quartz microbalance; and the fourth was assayed for Mg-ATPase. Each slice for enzyme assay was placed in a cold microtube. The tube was warmed slightly, allowing the section to thaw and adhere to the bottom. The tubes were stored at 4° for 2 hr in a desiccator prior to assay.

The total cortical thickness and the thicknesses of the cytoarchitectonic layers in each cylinder were estimated first by examination of the horizontal Nissl sections and then by direct measurements upon vertical sections cut from the block of cortex remaining after removal of the cylinder. This block of tissue was fixed in 10% formalin and frozen sections were cut adjacent to the site of the cylinder and parallel to its long axis. In these sections, stained with reduced silver, measurements were made using a calibrated microscope stage. Minimal shrinkage occurs with this histological technique. By correlation of the measurements in the silver preparation with the known depths of the horizontal Nissl sections in which the layers could be identified, reasonable estimates could be made of the cytoarchitectonic localizations of the samples assayed. The enzyme activities could, therefore, be plotted for each cortex in relation to the subpial depths and to the observed widths of the cortical laminae. The average cortical thickness was 2900 μ and the distance between successive Ca-ATPase (or Mg-ATPase) samples was 160 μ. Hence, for the assay of each enzyme, 17–24 samples were obtained in each experiment.

Ca-ATPase and Mg-ATPase activities were determined as described previously (HESS and POPE, 1959). The final concentrations of reagents in the Ca-ATPase assay were as follows: 0·005 M-Na_2ATP; 0·030 M-NaCl; 0·1 M-glycylglycine to give a final pH of 7·4; and 0·003 M-$CaCl_2$. The substrate solution for Mg-ATPase assays was the same except that 0·007 M-KCl was included and $CaCl_2$ was replaced by 0·007 M-$MgSO_4$. Of these solutions, 30 and 60 μl portions were used for the Ca-ATPase and Mg-ATPase determinations respectively. Samples were incubated exactly 15 min at 37°. Inorganic orthophosphate was estimated by a microadaptation of the method of FISKE and SUBBAROW (1925). The ATP used was either a 90 per cent pure non-chromatographed salt from Sigma Chemical Company, the chief impurity of which was ADP, or a chromatographed crystalline disodium salt from Pabst Chemical Company. All solutions for enzyme assays were prepared using glass redistilled water.

RESULTS

Ca-ATPase and Mg-ATPase activities (Tables 1 and 2) were measured at successive subpial depths in the biopsy specimens of human anterior frontal cortex. Mean activities were calculated for each depth sampled (series means) and for the cytoarchitectonic layers and sublayers (layer means) whose thicknesses in the different cortices were determined by examination of the accompanying histological material.

As the critical studies of LASHLEY and CLARK (1946) emphasized, large individual variations in laminar thicknesses, cell densities, and perikaryal areas may occur in the same region of cortex in a given species. Such variations mean that neither the subpial depth nor the cortical layer or sublayer alone is ideal as a territorial reference. Both were employed, the first because of its greater objectivity, the second because

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TABLE 1.—CA-ATPASE ACTIVITY IN RELATION TO CYTOARCHITECTURE OF HUMAN FRONTAL ISOCORTEX

Series	Depth (μ)	Layer	Ca-ATPase activity (micromoles P released/mg dry wt/hr)											
			Expt. H-34	Expt. H-36	Expt. H-37	Expt. H-38	Expt. H-39	Expt. H-40	Expt. H-51	Expt. H-52	Expt. H-53	Series Mean	Layer Mean*	Layer S.E.M.†
1	100	I	1·43	1.52	1·42	1·60	1·42	1·03	1·17		3·15	1·59	1·64	0·23
2	260	II	1·34	2·31		1·38	1·59	1·56	1·20	1·65	1·68	1·59	1·45	0·06
3	420		1·08	1·46	1·41	1·47		1·08	1·04	1·64		1·31		
4	580	(a)	1·33		1·47	1·55	1·33	1·66	0·94	1·52		1·40	1·33	0·06
5	740			0·96	1·43	1·03	1·28	1·37	1·37	1·39	1·57	1·30		
		III (b)											1·37	0·09
6	900		1·43	1·51	1·34	1·43	1·55	1·56	0·93	1·42	2·18	1·48		
7	1060	(c)	1·03	1·37		1·18	1·28	1·30	0·88	1·42	1·14	1·20	1·19	0·07
8	1220		0·94	1·64	1·32	1·35	1·09	0·72	0·98		1·79	1·23		
9	1380	IV	0·84	0·98	1·17		1·19		0·62	1·33	1·25	1·05	1·13	0·10
10	1540		1·19	0·94	1·60	1·13	1·27	0·98	0·89	1·20	1·50	1·19		
11	1700	V		1·24	1·28	0·93	1·00	1·07	0·70	1·20	1·35	1·10	1·11	0·07
12	1860		0·72	1·10	1·11	1·01	1·10	1·08		1·55	1·33	1·13		
13	2020		0·67	0·87	0·82	0·96	0·86	0·98	0·72	1·34	1·17	0·93		
		(a)											0·79	0·09
14	2180		0·66	0·89	0·63	1·00		0·90	0·65	1·38	1·44	0·94		
15	2340		0·36	0·62	0·34	0·56	0·64	0·78	0·61	1·33	0·94	0·69		
		VI (b)											0·44	0·07
16	2500				0·30		0·39	0·63		1·26		0·65		
17	2660		0·27	0·48	0·12	0·15	0·41	0·38	0·41	1·20		0·43		
18	2820		0·26	0·51		0·09	0·28	0·37	0·22	0·93	0·92	0·45		
19	2980	White matter		0·26			0·25		0·30		0·53	0·34		
20	3140			0·28				0·40	0·30	0·80		0·45		
21	3300			0·38				0·28		0·71		0·46		
22	3460			0·29				0·29				0·29		
23	3620							0·20				0·20		
24	3780							0·35				0·35		
Mean for cortex:			1·00	1·05	1·18	1·12	1·04	1·03	0·77	1·29	1·46	1·10 ± S.E.M. 0·07		

The dark horizontal lines indicate division of the cortex into layers and sublayers, based on examination of the histological control material for each experiment.

* Mean layer value. For each biopsy specimen, the determinations within each layer or sublayer were combined to give an average laminar value. From these individual average laminar values, the layer mean and its standard error (S.E.M.) were calculated for the entire experimental group.

† The S.E.M. evaluates, in combination, the variations in the biochemical assay and sampling procedure as well as biological variation within the population studied, and does not indicate the S.E.M. for the biochemical assay as such. The reasons for the high S.E.M. in these experiments have been discussed at some length in a previous article (HESS and POPE, 1959).

TABLE 2.—MG-ATPASE ACTIVITY IN RELATION TO CYTOARCHITECTURE OF HUMAN FRONTAL ISOCORTEX

Series	Depth (μ)	Layer	Mg-ATPase activity (μmoles P released/mg dry wt/hr)											
			Expt. H-34	Expt. H-36	Expt. H-37	Expt. H-38	Expt. H-39	Expt. H-40	Expt. H-51	Expt. H-52	Expt. H-53	Series Mean	Layer Mean*	Layer S.E.M.*†
1	180	I	5·52	3·79	8·77	8·20	5·41	5·48	6·41		7·89	6·43	6·80	0·46
2	340	II	4·81	7·15	9·78	8·49	7·87	9·30	10·66	6·97	6·13	7·91	7·98	0·69
3	500		5·57	7·73	7·82	8·40		8·70	9·04	5·22		7·50		
		(a)											6·79	0·52
4	660		5·96		6·62	8·01	7·95	6·89	7·65	5·16	7·52	6·97		
5	820			3·90	8·36	5·77	9·03	6·14	5·99	5·41	5·30	6·24		
		III (b)											7·11	0·60
6	980		6·81	7·29	8·77	8·59	9·43	5·12	8·78	5·55	4·05	7·15		
7	1140	(c)	5·97	4·85		8·20	8·09	6·89	7·82	5·33	4·68	6·48	7·15	0·71
8	1300	IV	6·53	4·02	9·52	9·13	8·60		11·82		4·26	7·70	7·29	0·62
9	1460		6·32	6·51	7·21		8·49	10·80	8·05	6·40	4·08	7·23		
10	1620		5·31	6·40	8·02	8·42	7·65	6·37	7·22	5·97	4·64	6·67		
		V											6·67	0·34
11	1780		5·12	6·88	7·61	7·55	7·19	5·84	9·58	5·21	6·36	6·82		
12	1940		5·01	7·93	7·44	6·98	6·72	9·06		5·92	5·15	6·78		
13	2100	(a)	4·18	6·62	6·72	7·42	6·33	7·72	6·33	5·93	3·77	6·11	6·07	0·41
14	2260	VI	3·74	4·36	5·62	8·10	7·04	6·13	7·20	5·71	5·44	5·93		
15	2420	(b)	3·35	6·33	3·33	6·97		5·26	6·36	4·98	3·23	4·98	3·63	0·33
16	2580				2·52		4·31	3·69		4·75	3·24	3·70		
17	2740		1·73	4·49	1·68	2·86	3·51	3·66	3·66	4·40		3·25		
18	2900		2·14	4·32		2·12	3·13	2·60	2·36	3·44	1·69	2·73		
19	3060	White matter		3·87		2·15	2·53		2·88		1·34	2·55		
20	3220			2·84		1·81		2·05		2·95		2·41		
21	3380			2·52				2·31		2·84		2·56		
22	3540			2·46				2·27				2·37		
23	3700							2·05				2·05		
24	3860							1·93				1·93		
Mean for cortex:			5·30	5·67	7·87	7·87	6·92	6·45	7·17	5·25	4·84	6·37 ± S.E.M. 0·39		
Ca-ATPase/Mg-ATPase			0·19	0·19	0·15	0·14	0·15	0·16	0·11	0·25	0·30	Mean = 0·18		

* See footnote Table 1. † See footnote Table 1.

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of its convenience and wide use. Because of the problem of biological variation, the application of rigid statistical analysis to the results of a relatively small number of experiments of this kind has limited value, as has been discussed previously (HESS and POPE, 1959, 1960). Consequently, a quite simple method was applied to examination of the data. The intracortical pattern obtained for each specimen has been considered both individually and in comparison with that of every other specimen and with the pattern of mean activity. Despite variations in the absolute values for enzyme activity from specimen to specimen as well as variations in the depth at which changes in activity occur, a clear resemblance has usually been apparent in the intracortical patterns in a group of such experiments.

In human cortex, as in rat cortex, the enzymic hydrolysis of ATP was much more strongly activated by Mg^{++} than by Ca^{++} The mean rate of Ca-ATPase activity was only 18 per cent of that of Mg-ATPase activity. The ratio of Ca-ATPase/Mg-ATPase activity in the series of biopsy specimens was not constant but varied from 0·11 to 0·30 (Table 2).

The mean activities of both enzymes at standard subpial depths are shown (Fig. 1) in relation to the mean widths of the laminae in the series of experiments.

The overall pattern of Ca-ATPase activity was characterized by a gradual decrease as a function of subpial depth, up to layer VI in which the decrease with depth was much greater. The mean activity in white matter, sampled in six of the nine experiments, was only 22 per cent of the mean activity in cortex and was significantly less than that in any cortical layer or sublayer. Mean activity in layer VI was significantly different from that in any of the other layers. The highest value for Ca-ATPase was found in layer I in five of the eight experiments in which this layer was sampled; in one of the five, the peak in layer III(b) was equally high. In two of the remaining experiments the highest value occurred in layer III(a), while in the third it was in layer IV. In the ninth instance, the highest values were recorded in layer II and layer III(a). In layer III, values in sublayers (a) and (b) tended to be higher than in sublayer (c) in all experiments. Values in layer IV were lower than those in the supragranular layers in seven of the eight experiments in which layer IV was sampled. Values in layer V were similar to those in layer IV. Finally, in all experiments, values in layer VI(a) were higher than those in VI(b). Thus, to a large extent, the features illustrated in the composite curve are characteristic of the individual experiments.

Mg-ATPase activity was well-sustained throughout the upper five layers of cortex, then decreased rapidly through layer VI to values in white matter which were one third to one fourth of the average values in cortical grey matter. When the results were combined according to their observed histological composition (layer means, Table 2), the highest values were found in layers II, III(b), III(c), and IV, although none of these layers was statistically different from its immediately adjacent layers. Each of the first five layers was significantly different in activity from both layer VI and white matter.

Values for Mg-ATPase activity in layer I were exceeded by those in layer II (or the border of layers I and II) in six of eight experiments, and by one or more values in layer III in seven of eight experiments. In six of nine experiments, values in layer II were higher than those in the adjacent part of layer III. In layer III the locus of maximum activity was variable; in four experiments it was in sublayer (b), in three in sublayer (c), and in two in sublayer (a). In four of the six experiments in which the

border of layer III(c) with layer IV was sampled, the former was higher than the latter. In six experiments the activity in layer IV was higher than that in the adjacent part of layer V; but in three instances the value in the latter was greater. The mean value for

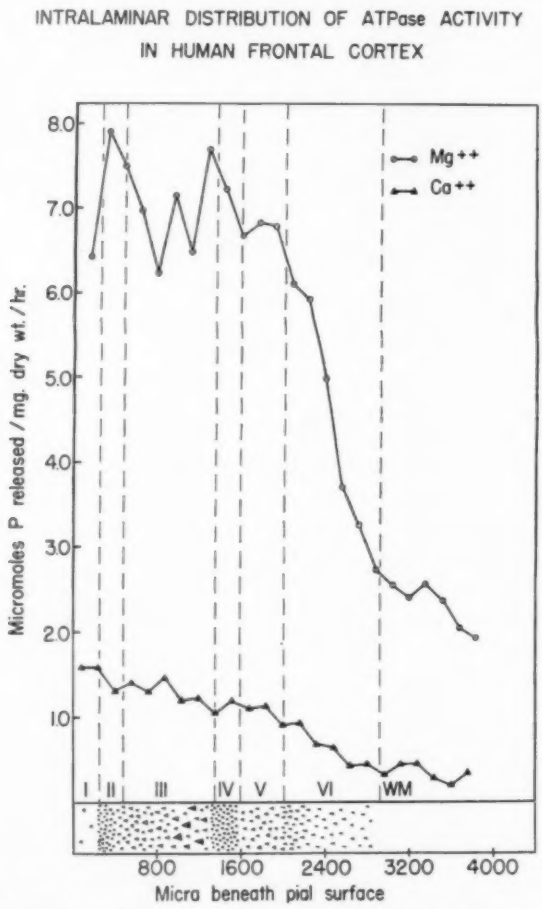

FIG. 1.—Mg-ATPase and Ca-ATPase activities are plotted in relation to subpial depths and cytoarchitecture. In this series of biopsy specimens, average cortical thickness was 2910 μ. Layer I averaged 8·8%; II, 7·9%; III, 30·1%; IV, 8·4%; V, 14·2%; and layer VI, 30·7% of total width.

layer IV (Table 2) is greater than that for sublayer III(c), but when the values are averaged according to depth (series means) and average layer widths, the higher value is in layer III(c) as it borders layer IV. This suggests that exceptionally high Mg-ATPase activity resides in structures at the junction of these layers or in the upper part of layer IV. In all experiments values in layer VI(a) were higher than those in VI(b) and in five experiments a small peak of activity occurred in VI(a).

In summary, Ca-ATPase activity is highest in layers I and II and gradually decreases as a function of increasing subpial depth. Small peaks of activity tend to occur in layer III. Mg-ATPase activity, on the other hand, although well-sustained

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throughout the first five layers, tends to be highest in layers II, III(b), III(c), and IV. In the case of both enzymes, values in white matter are only about one fourth of the average values in the cortex.

DISCUSSION

The results will be discussed first with respect to the specificity of the assay methods and the localization of ATPases in tissue preparations obtained by centrifugation of homogenates; second, with respect to cortical histological structure. Finally, the mean cortical ATPase activities will be compared with results reported by other investigators.

In tissue slices Ca- and Mg-activated ATPases clearly cannot be assayed completely independently. Ca^{++} activates Mg-ATPases only slightly or not at all, so that Ca-ATPase activity may indicate the presence of myosin-like proteins. Mg-ATPase activity however, may represent a composite of different enzymes. First, four Mg-stimulated ATPases have been distinguished in mitochondria, at least in liver and muscle (HÜLSMANN and SLATER, 1957); it has been suggested that three of these enzymes are transphosphorylases concerned with oxidative phosphorylations, ATPase activity resulting from a reversal of their normal function. Another view, however, is that 'unmasking' of ATPase activity merely reflects the fact that ATP substrate has been made available to internal ATPase by a change in mitochondrial permeability (GREEN, 1959). In addition, microsome-sized particulate preparations of Mg-ATPase have been obtained from muscle (KIELLEY and MEYERHOF, 1948), brain (BINKLEY and OLSON, 1950) and nerve (SKOU, 1957); the origin of such ATPases may be from the endoplasmic reticulum. Furthermore, Mg-ATPase activity may include some actomyosin ATPase activity, which at low ionic strengths of KCl is stimulated by Mg^{++} (BOWEN, 1957).

It is apparent from Tables 1 and 2 and Fig. 1 that Ca-ATPase and Mg-ATPase activities are not due to the same enzyme or enzymes, although some overlap is not excluded. For both ATPases, as for every other enzyme studied so far, the patterns of distribution in human frontal cortex parallel those previously determined in rat somaesthetic cortex to a remarkable extent, despite the species and areal differences in cortical cytoarchitecture and in absolute levels of enzyme activity.

The pattern of Ca-ATPase activity in human cortex resembles not only that of the same enzyme in rat cortex but also that observed for AChE in human cortex (POPE *et al.*, 1952). This is not surprising, since the distributions of Ca-ATPase and AChE are similar in rat cortex; however, three of the features shown by AChE in the granular and infragranular layers are not apparent in the distribution of Ca-ATPase in either rat or human cortex. These are: a marked valley of activity in layer IV; a definite peak of activity in layer V; and a small peak in layer VI near the border with white matter. In comparison, Ca-ATPase activity is only slightly lower in layer IV than in the supragranular layers; values in layer V only occasionally exceed those in layer IV; and no rise in activity occurs at the border of layer VI and white matter. High values for Ca-ATPase in the upper part of layer I in both rat and human cortex may be due in part to the occurrence of cortical surface vessels which, in rare instances, were present in the first slice. The possible contributions of blood vessels and of neuroglia to the intracortical patterns of ATPases were discussed previously (HESS and POPE, 1959).

The intracortical pattern of AChE has been analysed with respect to what is known of the protoplasmic and fibrillar fine structure of rodent cortex (POPE, 1952) and human cortex (POPE *et al.*, 1952), with the conclusion that the distribution of AChE is consistent with its association principally with the finer ramifications of axons and dendrites in the cortex, including synaptic junctions. Since it is generally similar to AChE in distribution, Ca-ATPase activity presumably is localized in much the same structures. Possibly, AChE and Ca-ATPase may be related to the same general type of subcellular structure but show slightly different distributional features because of a greater concentration of AChE in cholinergic neurons. Recently GIACOBINI (1959), using the cartesian diver ultramicro-respirometer, demonstrated in spinal ganglia three groups of neurons having respectively high, low, and no AChE activity. Of cell parts studied in this way (GIACOBINI, 1960), the cell body had the highest activity in terms of volume; dry weight values were not reported. Axons and dendrites ranked next in activity, while little activity was found in nuclei and none in nucleoli. This work has been extended to anterior horn cells of spinal cord but not as yet to neurons of cerebral cortex.

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The apparent localization of Ca-ATPase activity in plexuses of axons and dendrites suggests that a myosin-like contractile protein may reside in surface membranes and participate in the permeability changes during neuronal excitation, as originally proposed by LIBET (1948). Interestingly, the ACh receptor protein is postulated to perform such a function by a change in its configuration (NACHMANSOHN, 1959). One of the other proteins involved in ACh metabolism, namely AChE, has been reported to show ATPase activity (BERNHARD, 1955), while myosin shows AChE activity (VARGA *et al.*, 1955).

The intracortical distribution of Mg-ATPase in human cortex, besides being very similar to that of this enzyme in rat cortex (HESS and POPE, 1959), shows points of resemblance to the distributions of cytochrome oxidase in human cortex (HESS and POPE, 1960) and of the oxidase and NAD in rat cortex (POPE, HESS, WARE and THOMSON, 1956). The distributions of cytochrome oxidase and NAD were interpreted as indicating their localization predominantly in neuronal somata, dendrites, and axon terminals. The generally similar pattern of Mg-ATPase seems consistent with localization of this enzyme also at these sites, although lesser activity (in terms of dry weight) may well be found in axons and neuroglia. In human cortex the distribution of Mg-ATPase differed from that of cytochrome oxidase mainly in the relative height of the peak in layer III(b). While cytochrome oxidase was most active in layer III (b), Mg-ATPase tended to be greater at the junctions of layers I and II and of layers III(c) and IV. Presumably, the distribution of Mg-ATPase activity resembles that of cytochrome oxidase activity to the extent that both are present in mitochondria, but differs because other cell membranes having ATPase activity are not distributed proportionally with mitochondria. The functional role of Mg-ATPase activity in brain is at present speculative. Recent work has tended to support the idea that an enzyme of this sort may be involved in the process of active Na^+ transport, since it is affected in a characteristic way by Na^+ and K^+ ions (HESS and POPE, 1957; SKOU, 1957, 1960; MCILWAIN and BALAKRISHNAN, 1961).

Because of the histological complexity of the cortex, analyses upon samples of the size described in this article cannot definitely establish the cytological localization of a constituent. For this purpose, it is necessary either to sample and analyse single cells

and cell parts directly (LOWRY, 1957; PASSONNEAU and LOWRY, 1961; ROBINS, 1960; HIRSCH and ROBINS, 1961; BRATTGÅRD *et al.*, 1957) or to visualize the localization by means of classical histochemistry. The careful use of histochemical stains, as in studies of AChE (KOELLE, 1955; KOELLE and KOELLE, 1959) with the light microscope and of ATPase activity (ESSNER *et al.*, 1958; TICE and BARRNETT, 1960) with the electron microscope, can provide information otherwise unobtainable. Eventually such techniques will reveal precise sites of localization of ATPases and other enzymes in cortical tissue.

The mean Ca-ATPase and Mg-ATPase activities in human cortex are 50 per cent and 80 per cent of the respective mean values in rat cortex. These differences in activity between the two species are less than those encountered with most of the other enzymes studied so far, but similar to the difference in their average rates of cortical respiration *in vitro*. AChE activity in human cortex averages 35 per cent, cytochrome oxidase 10 per cent, and L-alanylglycine dipeptidase 200 per cent of the mean activity in rat cortex (POPE, 1952; POPE *et al*, 1956; HESS and POPE, 1959, 1960). Curiously, the average rate of *in vitro* respiration in human cortex is 50 per cent of that in rat cortex (ELLIOTT, 1948), in spite of the fact that its cytochrome oxidase activity is only 10 per cent as great.

The maximum rate of hydrolysis of ATP in human cortex is greater than the probable rate of resynthesis of the compound by oxidative phosphorylation, if a P/O ratio of three is assumed. The mean Mg-ATPase activity of human frontal cortex is equivalent to $1{\cdot}08 \times 10^{-3}$ g-atoms P/g fresh wt/hr at 37°, while the mean oxygen uptake is 2·20 ml/g fresh wt/hr (HESS and POPE, 1960), or $0{\cdot}196 \times 10^{-3}$ g-atoms O/g fresh wt/hr at 37°. The rate of breakdown of ATP, therefore, might under some conditions exceed its rate of resynthesis by a factor of almost two. Possibly, current *in vitro* estimates of the rate of respiration of human cortex are low, so that the rate of ATP resynthesis may be greater *in vivo*.

PAPPIUS and ELLIOTT (1954*a*, *b*) determined Ca-ATPase and Mg-ATPase activities in rat and human brain suspensions. Their results for Ca-ATPase activity in rat and human cortex respectively are about twice those reported here. Mg-ATPase values for rat cortex are essentially the same by their technique and ours. However, our average values for Mg-ATPase activity in anterior frontal cortex from patients with mental illness exceed by about 80 per cent their values for the enzyme in temporal and parietal cortex from epileptic and non epileptic neurosurgical patients. In view of the reports that some of the tranquilizing drugs, such as chlorpromazine, affect Mg-ATPase activity (LÖW, 1959*a*, *b*) it should be stated that the patients studied had not received these drugs. For a number of reasons, however, the results may not be strictly comparable. First, the two assay methods for Mg-ATPase may have given rise to different results in human tissue, even though they did not in rat tissue; differences in ATP preparations (identity of impurities) and in concentrations of Na^{+}, K^{+}, Ca^{++}, and Mg^{++} in the assay media (contributed in part by the tissue itself) may have existed. Second, it is possible that human frontal areas have greater Mg-ATPase activity than do temporal and parietal areas; in the monkey, the activity in visual cortex is 33 per cent greater than in motor cortex (ROBINS *et al.*, 1956). Whether the observed difference is related in any way to mental disease cannot be decided, therefore, without further investigation.

SUMMARY

Quantitative microchemical techniques have been used to determine the distributions of Ca-ATPase and Mg-ATPase activities within the cytoarchitectonic layers of human anterior frontal cortex in biopsy specimens excised during lobotomies.

Ca-ATPase activity per unit dry weight is greatest in layers I and II and thereafter declines as a function of the subpial depth. The activity in white matter is one fifth of the mean activity in the cortex. The distribution is consistent with localization of Ca-ATPase activity principally in the finely subdivided plexuses of axons and dendrites in the cortex.

Mg-ATPase activity per unit dry weight is well-sustained throughout layers I—V inclusive, with slightly higher values being found in layers II, III(b), III(c), and IV. The activity decreases rapidly through layer VI to values in white matter which are a quarter of the average value in the cortex. This intralaminar pattern is consistent with the occurrence of Mg-ATPase activity to an important extent in neuronal somata, dendrites, and axon terminals.

Mean cortical Ca-ATPase activity is only 18 per cent of the mean Mg-ATPase activity.

Following the trend shown by other enzymes studied, Ca-ATPase and Mg-ATPase have intracortical distribution patterns in human cortex which parallel their respective distributions in rat cortex. The results support the idea that Ca^{++} and Mg^{++} activate enzymes which are localized predominantly in different histological sites and which mediate different functions in nervous tissues.

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PRELIMINARY NOTE

Guanase activity in brain

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PURINES are believed to be catabolized mainly in the liver. It has, however, been observed that one of the enzymes concerned in purine catabolism, guanase, is active in the brain.

In the present experiments albino rats were decapitated and the liver was removed after perfusion with 0·25 M-sucrose at 3–5° through the hepatic vein. Blood clots were removed from the brain by rinsing in cold saline (3–5°). Weighed portions of both organs were homogenized in 10 vol of twice distilled water in a Potter-Elvehjem type of homogenizer using a teflon pestle. The homogenate was centrifuged for 15 min at 17,000 **g** in a refrigerated centrifuge at 3°. The supernatant fluid, which was used for enzyme assays, was shown previously to contain all the guanase activity of the tissue (LAMIRANDE, ALLARD and CANTERO, 1958). The method used for enzyme assay is a modification of the procedure suggested by ROUSH and NORRIS (1950). The decrease in optical density at 248 mμ (the maximum for guanine) was taken as a measure of the substrate metabolized. The incubation mixture consisted of 0·3 ml of 0·1 M-glycine buffer, pH 9·0; 0·6 ml of 0·001 M-guanine (CfP); and 0·1 ml of tissue homogenate. After incubation for 5 min at 37°, 1 ml of 10% (w/v) perchloric acid was added to stop the reaction. The volume was made up to 3 ml with water; the contents were mixed and centrifuged. The optical density of the supernatant was read in a Hilger Uvispek Spectrophotometer. Duplicate enzyme assays were made. Appropriate substrate and enzyme blanks were taken in each series. The protein content of the enzyme preparation was obtained by the method of WARBURG and CHRISTIAN (1941). DNA was estimated by the method of SCOTT, FRACASTORO and TAFT (1956).

TABLE 1.—GUANASE ACTIVITY IN RAT BRAIN AND LIVER

Rat no.	Specific activity (μmoles guanine degraded in 5 min/mg protein)		Rat no.	Specific activity (μmoles guanine degraded per min per 100 μg tissue DNA)	
	Brain	Liver		Brain	Liver
1	6·0	1·7	5	31·1	14·7
2	5·5	3·2	6	25·6	9·3
3	4·3	2·1	7	10·3	6·3
4	6·3	3·0	8	13·2	6·6
Mean ± S.D.	5·5 ± 0·88	2·5 ± 0·71	Mean	20·0 ± 9·18	9·2 ± 3·89

TABLE 2.—DISTRIBUTION OF GUANASE ACTIVITY IN VARIOUS ORGANS OF RAT

	Liver	Brain	Kidney	Heart	Lungs	Spleen
Guanase activity (μmoles guanine degraded/min/100 μg DNA)	10·1	27·0	9·3	12·3	6·7	3·7

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Since the cell density was likely to vary from organ to organ, comparison of the enzyme activity in different organs is made on the basis of the DNA content as well as the protein content of the tissue. Data for specific activity of the enzyme (Table 1) show that the activity in brain is twice as high as in liver. This also applies when the activity is expressed on the basis of tissue DNA. The distribution of the enzyme in other organs of the rat is shown in Table 2.

The function of this enzyme in the brain is undefined. SCHMITZ, HURLBERT and POTTER (1954) and, later, THOMAS (1957) reported the presence of guanine nucleotides in extracts of rat brain. THRELFALL (1957) noted that guanine nucleotides constitute 18–20 per cent of the total nucleotides of the brain, whereas in muscle they constitute only 2–3 per cent of the total nucleotides. HEALD (1957) observed that the guanine nucleotides in brain tissue slices incubated in a medium containing ^{32}P increased in specific radio-activity when electrical impulses were applied. A breakdown of RNA has been found to occur in the anterior horn cells as a result of stimulation (HYDEN, 1943). VRBA and FOLBERGROVA (1959) observed a decrease in the protein and nucleic acid content of the brain in rats during strenuous physical exercise. GEIGER (1957) reported that the composition of the nucleic acids in the cat brain alters during metrazol convulsions, the cytidine and adenine contents of the nucleic acids increasing in proportion to the uridine and guanine contents. RAPPOPORT, FRITZ, ALLEN and MORACZEWSKI (1960) have recently reported the existence of an active guanosine phosphorylase in the brain. The evidence is still insufficient to assign any functional role to the guanase in the brain.

Department of Biochemistry
All-India Institute of Medical Sciences
New Delhi-16.

G. P. TALWAR
B. K. GOEL
M. MANSOOR
N. C. PANDA

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Papers to be published in future issues

S. I. SINGH and D. L. MALHOTRA: Amino acid content of monkey brain—I. General pattern and quantitative values of glutamic acid, glutamine γ-aminobutyric acid and aspartic acid.

P. HOROWICZ and M. G. LARRABEE: Oxidation of glucose in a mammalian sympathetic ganglion at rest and in activity.

S. S. HOTTA: Glucose metabolism in brain tissue. The hexosemonophosphate shunt and its role in glutathione reduction.

E. DE ROBERTS *et al.*: Cholinergic and noncholinergic nerve endings in rat brain—I. Isolation and subcellular distribution of acetylcholine and acetylcholinesterase.

G. WIEGAND and E. SCHERFLING: Determination of 5-hydroxytryptophan and serotonin.

N. KARKI, R. KUNTZMAN and B. B. BRODIE: Storage, synthesis and metabolism of monoamines in the developing brain.

M. WOLMAN: Extraction of complexes from central and peripheral myelin of man.

H. E. HIRSCH and E. ROBINS: Distribution of γ-aminobutyric acid in layers of the cerebral and cerebellar cortex. Implications for its physiological role.

F. GOTOH *et al.*: Sodium and potassium of blood and brain.

J. DURELL and P. J. HEALD: Potassium ions and brain phosphates.

R. L. POTTER and A. VAN HARREVELD: The effect of metrazol on the glutamate metabolism of cerebral cortex.

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